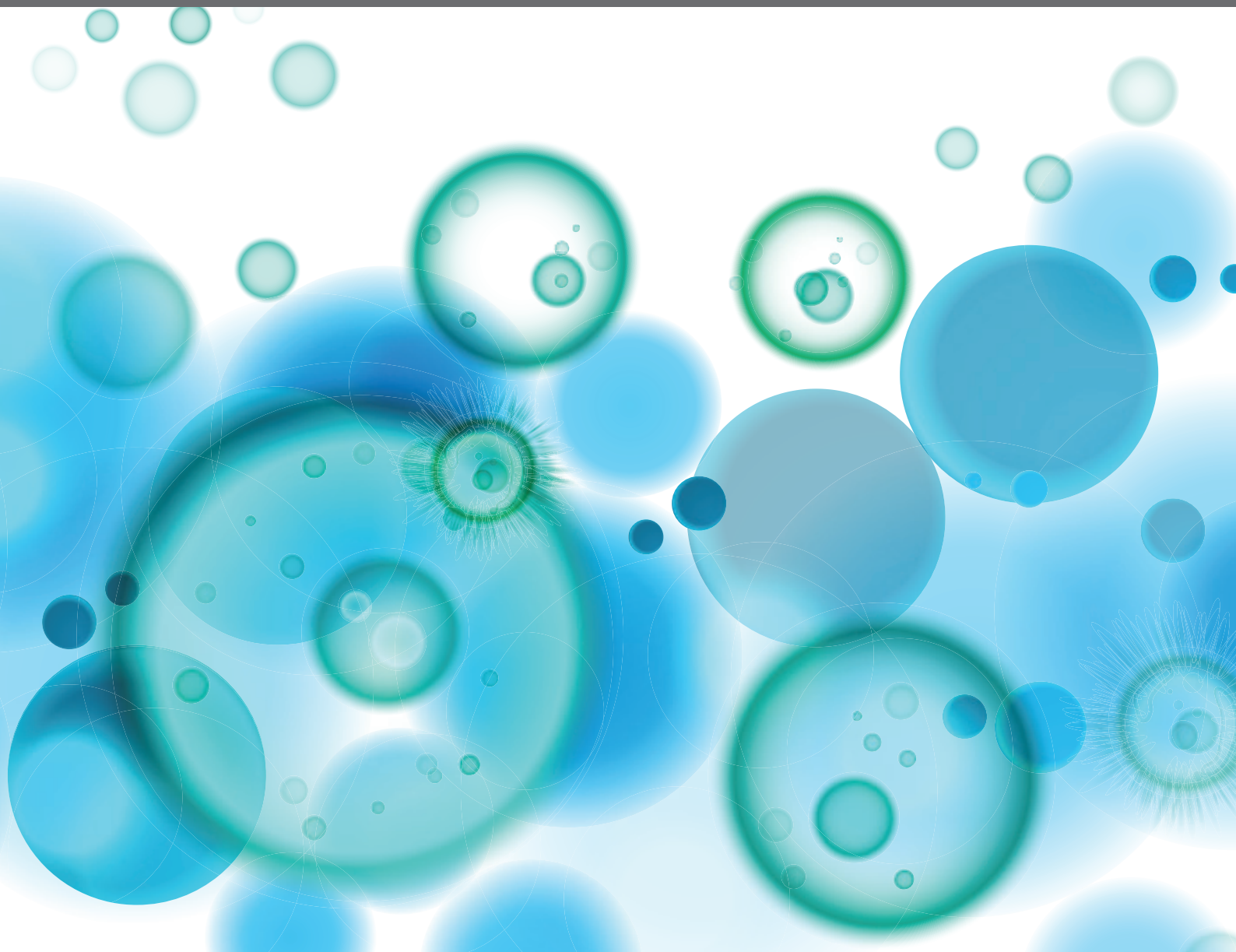


FOLLICULAR HELPER T CELLS IN IMMUNITY AND AUTOIMMUNITY

EDITED BY: Georgia Fousteri, Shahram Salek-Ardakani and Maria Pia Cicalese
PUBLISHED IN: *Frontiers in Immunology*





frontiers

Frontiers eBook Copyright Statement

The copyright in the text of individual articles in this eBook is the property of their respective authors or their respective institutions or funders. The copyright in graphics and images within each article may be subject to copyright of other parties. In both cases this is subject to a license granted to Frontiers.

The compilation of articles constituting this eBook is the property of Frontiers.

Each article within this eBook, and the eBook itself, are published under the most recent version of the Creative Commons CC-BY licence.

The version current at the date of publication of this eBook is CC-BY 4.0. If the CC-BY licence is updated, the licence granted by Frontiers is automatically updated to the new version.

When exercising any right under the CC-BY licence, Frontiers must be attributed as the original publisher of the article or eBook, as applicable.

Authors have the responsibility of ensuring that any graphics or other materials which are the property of others may be included in the CC-BY licence, but this should be checked before relying on the CC-BY licence to reproduce those materials. Any copyright notices relating to those materials must be complied with.

Copyright and source acknowledgement notices may not be removed and must be displayed in any copy, derivative work or partial copy which includes the elements in question.

All copyright, and all rights therein, are protected by national and international copyright laws. The above represents a summary only. For further information please read Frontiers' Conditions for Website Use and Copyright Statement, and the applicable CC-BY licence.

ISSN 1664-8714

ISBN 978-2-88963-847-5

DOI 10.3389/978-2-88963-847-5

About Frontiers

Frontiers is more than just an open-access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers Journal Series

The Frontiers Journal Series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the Frontiers Journal Series operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to Quality

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews. Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view. By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the Frontiers Journals Series: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area! Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers Editorial Office: researchtopics@frontiersin.org

FOLLICULAR HELPER T CELLS IN IMMUNITY AND AUTOIMMUNITY

Topic Editors:

Georgia Fousteri, San Raffaele Hospital (IRCCS), Italy

Shahram Salek-Ardakani, Pfizer (United States), United States

Maria Pia Cicalese, San Raffaele Scientific Institute (IRCCS), Italy

Citation: Fousteri, G., Salek-Ardakani, S., Cicalese, M. P., eds. (2020). Follicular Helper T Cells in Immunity and Autoimmunity. Lausanne: Frontiers Media SA.
doi: 10.3389/978-2-88963-847-5

Table of Contents

- 06 Editorial: Follicular Helper T Cells in Immunity and Autoimmunity**
Maria Pia Cicalese, Shahram Salek-Ardakani and Georgia Foustari
- 11 The Janus Face of Follicular T Helper Cells in Chronic Viral Infections**
Ute Greczmiel and Annette Oxenius
- 26 Relapsing–Remitting Multiple Sclerosis is Characterized by a T Follicular Cell Pro-Inflammatory Shift, Reverted by Dimethyl Fumarate Treatment**
Vanessa Cunill, Margarita Massot, Antonio Clemente, Carmen Calles, Valero Andreu, Vanessa Núñez, Antonio López-Gómez, Rosa María Díaz, María de los Reyes Jiménez, Jaime Pons, Cristófol Vives-Bauzá and Joana Maria Ferrer
- 39 Correlates of Follicular Helper Bias in the CD4 T Cell Response to a Retroviral Antigen**
Luca Danelli, Tiziano Donnarumma and George Kassiotis
- 54 Unexpected Help: Follicular Regulatory T Cells in the Germinal Center**
Markus M. Xie and Alexander L. Dent
- 63 T Follicular Helper Cells in Autoimmune Disorders**
Noémie Gensous, Manon Charrier, Dorothée Duluc, Cécile Contin-Bordes, Marie-Elise Truchetet, Estibaliz Lazaro, Pierre Duffau, Patrick Blanco and Christophe Richez
- 81 Regulation of T Follicular Helper Cells in Islet Autoimmunity**
Isabelle Serr and Carolin Daniel
- 88 T Follicular Helper-Like Cells in Inflamed Non-Lymphoid Tissues**
Andreas Hutloff
- 93 Follicular Helper T Cells in DiGeorge Syndrome**
Adam Klocperk, Zuzana Paračková, Markéta Bloomfield, Michal Rataj, Jan Pokorný, Susanne Unger, Klaus Warnatz and Anna Šedivá
- 102 Posttranscriptional Gene Regulation of T Follicular Helper Cells by RNA-Binding Proteins and microRNAs**
Dirk Baumjohann and Vigo Heissmeyer
- 111 Follicular Helper T Cells in Systemic Lupus Erythematosus**
Sun Jung Kim, Kyungwoo Lee and Betty Diamond
- 119 Several Follicular Regulatory T Cell Subsets With Distinct Phenotype and Function Emerge During Germinal Center Reactions**
Nicolas Fazilleau and Meryem Aloulou
- 128 Synaptic Interactions in Germinal Centers**
Ilenia Papa and Carola G. Vinuesa
- 138 Insights Into the Molecular Mechanisms of T Follicular Helper-Mediated Immunity and Pathology**
Lei Qin, Tayab C. Waseem, Anupama Sahoo, Shayahati Bieerkehazhi, Hong Zhou, Elena V. Galkina and Roza Nurieva
- 159 T Cells That Help B Cells in Chronically Inflamed Tissues**
Deepak A. Rao

- 172 ***Low Peripheral T Follicular Helper Cells in Perinatally HIV-Infected Children Correlate With Advancing HIV Disease***
Bret McCarty, Mussa Mwamzuka, Fatma Marshed, Matthew Generoso, Patricia Alvarez, Tiina Ilmet, Adam Kravietz, Aabid Ahmed, William Borkowsky, Derya Unutmaz and Alka Khaitan
- 184 ***Control of Germinal Center Responses by T-Follicular Regulatory Cells***
James B. Wing, Murat Tekgüç and Shimon Sakaguchi
- 196 ***T Cell/B Cell Collaboration and Autoimmunity: An Intimate Relationship***
Lina Petersone, Natalie M. Edner, Vitalijs Ovcinnikovs, Frank Heuts, Ellen M. Ross, Elisavet Ntavli, Chun J. Wang and Lucy S. K. Walker
- 206 ***Lymph Node Cellular Dynamics in Cancer and HIV: What Can We Learn for the Follicular CD4 (Tfh) Cells?***
Antigoni Poultisidi, Yiannis Dimopoulos, Ting-Fang He, Triantafyllos Chavakis, Emmanouil Saloustros, Peter P. Lee and Constantinos Petrovas
- 218 ***Determination of T Follicular Helper Cell Fate by Dendritic Cells***
Jayendra Kumar Krishnaswamy, Samuel Alsén, Ulf Yrlid, Stephanie C. Eisenbarth and Adam Williams
- 234 ***IL-21 Biased Alemtuzumab Induced Chronic Antibody-Mediated Rejection Is Reversed by LFA-1 Costimulation Blockade***
Jean Kwun, Jaeberm Park, John S. Yi, Alton B. Farris, Allan D. Kirk and Stuart J. Knechtle
- 245 ***Molecular Control of Follicular Helper T cell Development and Differentiation***
Haijing Wu, Yaxiong Deng, Ming Zhao, Jianzhong Zhang, Min Zheng, Genghui Chen, Linfeng Li, Zhibiao He and Qianjin Lu
- 256 ***Regulation of the Germinal Center Response***
Marisa Stebegg, Saumya D. Kumar, Alyssa Silva-Cayetano, Valter R. Fonseca, Michelle A. Linterman and Luis Graca
- 269 ***Control of the Germinal Center by Follicular Regulatory T Cells During Infection***
Brodie Miles and Elizabeth Connick
- 275 ***CCR9 Expressing T Helper and T Follicular Helper Cells Exhibit Site-Specific Identities During Inflammatory Disease***
Ilaria Cosorich, Helen M. McGuire, Joanna Warren, Mark Danta and Cecile King
- 290 ***PI3K Orchestrates T Follicular Helper Cell Differentiation in a Context Dependent Manner: Implications for Autoimmunity***
Silvia Preite, Bonnie Huang, Jennifer L. Cannons, Dorian B. McGavern and Pamela L. Schwartzberg
- 300 ***Molecular Basis of the Differentiation and Function of Virus Specific Follicular Helper CD4⁺ T Cells***
Qizhao Huang, Jianjun Hu, Jianfang Tang, Lifan Xu and Lilin Ye
- 311 ***Interleukin-1 in the Response of Follicular Helper and Follicular Regulatory T Cells***
Paul-Gydéon Ritvo and David Klatzmann

318 *P2RX7 Deletion in T Cells Promotes Autoimmune Arthritis by Unleashing the Tfh Cell Response*

Krysta M. Felix, Fei Teng, Nicholas A. Bates, Heqing Ma, Ivan A. Jaimez, Kiah C. Sleiman, Nhan L. Tran and Hsin-Jung Joyce Wu

331 *The Transcription Factor T-Bet is Required for Optimal Type I Follicular Helper T Cell Maintenance During Acute Viral Infection*

Pengcheng Wang, Youping Wang, Luoyingzi Xie, Minglu Xiao, Jialin Wu, Lifan Xu, Qiang Bai, Yaxing Hao, Qizhao Huang, Xiangyu Chen, Ran He, Baohua Li, Sen Yang, Yaokai Chen, Yuzhang Wu and Lilin Ye



Editorial: Follicular Helper T Cells in Immunity and Autoimmunity

Maria Pia Cicalese^{1,2}, Shahram Salek-Ardakani³ and Georgia Fousteri^{4*}

¹ San Raffaele Telethon Institute for Gene Therapy (TIGET), San Raffaele Scientific Institute, Milan, Italy, ² Pediatric Immunohematology and Bone Marrow Transplantation Unit, IRCCS San Raffaele Scientific Institute, Milan, Italy, ³ Department of Pathology, Immunology and Laboratory Medicine, University of Florida, Gainesville, FL, United States, ⁴ Division of Immunology Transplantation and Infectious Diseases (DITID), Diabetes Research Institute (DRI), IRCCS San Raffaele Scientific Institute, Milan, Italy

Keywords: follicular helper T cells, follicular regulatory T cells, autoimmunity, primary immunodeficiency, immunity, cancer, infection, transplantation

Editorial on the Research Topic

Follicular Helper T Cells in Immunity and Autoimmunity

In the last two decades, a new population of CD4⁺ helper T cells, named T follicular helper cells (Tfh), was shown to be specialized in “helping” the germinal center (GC) response and reported to play important roles in type I, II, and III immune responses. High expression of CXCR5 and low expression of CCR7 enable Tfh cells to enter and stay in GCs. In the light zone of GCs, Tfh cells provide crucial signals to antigen-specific B cells, promoting somatic hypermutation, class switch recombination (CSR), and affinity maturation through cellular interactions and cytokine secretion. In addition, Tfh cells also facilitate the differentiation of memory B cells and long-lived plasma cells. Tfh cells can be found in circulation and recent studies have identified multiple Tfh subsets based on the expression of chemokine receptors and activation molecules. The distribution of circulating Tfh subsets, their number and activation phenotype have been associated with the clinical outcome of numerous diseases spanning from autoimmunity to immunodeficiency and were shown to correlate with responses to infections and vaccines (1). Furthermore, a population of FOXP3⁺ regulatory T cells that expresses CXCR5 and controls GC responses, named follicular regulatory T cells (Tfr), was recently described and shown to suppress Tfh cell-mediated antibody responses. However, the factors that control their generation and the mechanisms of suppression are poorly understood (2).

Tfh and Tfr cells are currently being used as biomarkers and therapeutic targets in various clinical settings, including cancer and transplantation (3–13). A clear understanding of the mechanisms that control the development of Tfh and Tfr cells and the factors that contribute to GC responses is vital to the success of Tfh-based immune-monitoring and therapy development. This Special Research Topic aimed to address several questions with the final objective to understand better the biology of Tfh cells and how they could be targeted to harness undesired immune responses or boost immunity. Our Special Research Topic “Follicular Helper T Cells in Immunity and Autoimmunity” brought together several outstanding experts in the field. Here, we discuss the main messages from eight original research articles, one perspective and twenty state-of-the-art review articles these experts contributed divided into eight themes, as reported below.

OPEN ACCESS

Edited and reviewed by:

Barbara Fazekas De St. Groth,
University of Sydney, Australia

*Correspondence:

Georgia Fousteri
fousteri.georgia@hsr.it

Specialty section:

This article was submitted to
T Cell Biology,
a section of the journal
Frontiers in Immunology

Received: 13 December 2019

Accepted: 30 April 2020

Published: 29 May 2020

Citation:

Cicalese MP, Salek-Ardakani S and
Fousteri G (2020) Editorial: Follicular
Helper T Cells in Immunity and
Autoimmunity.
Front. Immunol. 11:1042.
doi: 10.3389/fimmu.2020.01042

MOLECULAR AND CELLULAR REGULATION OF Tfh CELL DEVELOPMENT AND DIFFERENTIATION

The development and differentiation of Tfh cells have been shown to be regulated by transcription factors such as the B-cell lymphoma 6 protein (Bcl-6), the signal transducer and activator of transcription 3 (STAT3), and the B lymphocyte-induced maturation protein-1 (Blimp-1). Also, cytokines, including IL-6 and IL-21, are essential for Tfh cell development. In the last step of Tfh cell commitment, B cells become the primary antigen-presenting cell and provide signals including ICOSL/ICOS, CD40/CD40L, and CD84/CD84-SAP that complete Tfh cell differentiation program (1). In their review article, Wu et al. summarize the recent advances in the molecular regulation of Tfh cell development and differentiation at the protein and epigenetic levels. Identification of the factors that instruct Tfh cell differentiation could increase the pipeline of potential targets for future clinical interventions.

On the other hand, Tfh cell dysregulation may result in aberrant antibody responses that frequently coincide with autoimmune disease or allergy development (4). The fate and identity of Tfh cells are tightly controlled by gene regulation at the transcriptional and post-transcriptional level. Baumjohann et al. provide a nice overview on the complex and dynamic regulatory network of post-transcriptional mechanisms that regulate Tfh cell differentiation, function, and plasticity through the actions of RNA-binding proteins (RBPs, belonging to the Roquin family) and small endogenously expressed regulatory RNAs called microRNAs (miRNAs). RBPs have been shown to dampen spontaneous activation and differentiation of naïve CD4⁺ T cells into Tfh cells, as evidenced by the propensity of Roquin-mutant CD4⁺ T cells to differentiate into Tfh cells providing inappropriate B cell help signals promoting the production of autoantibodies. Interestingly, Regnase-1, an endoribonuclease that regulates many molecules that are also targets of Roquin, was crucial for the prevention of autoantibody production (Baumjohann et al.).

Significant progress has been made in defining the potential of different dendritic cell (DC) subsets in supporting Tfh priming (Wu et al.). Krishnaswamy et al. propose that the location of different DC subsets within the lymph node (LN) and their access to antigen determines their potency in Tfh cell priming. Indeed, migratory cDC1s, resident cDC1s, and Langerhans cells (LCs) are found within the T cell zones. Migratory cDC2s are located in the T-B border region (including the interfollicular zone) and resident cDC2s reside in the lymphatic sinuses. The authors propose an interesting three-step model for Tfh cell differentiation involving diverse subpopulations of DCs: step 1: Antigen transport and naïve T cell activation (antigen presented by migratory cDCs, LN-resident cDC2s or transported by migratory DCs to resident DC subsets in the LNs); step 2: Pre-Tfh differentiation by migratory cDC2s at the T-B border; step 3: Tfh commitment by B cells (Krishnaswamy et al.).

A very elegant summary of the GC niche and the complex immunological synapses between Tfh and GC B cells are provided

by Papa and Vinuesa. According to the authors, Tfh:B cell cognate interactions need to be critically fast and short at the level of the GC for affinity-selection, where in B cells compete for T cell help so that rapid modulation of the signaling threshold determines the outcome of the interaction. Moreover, promiscuous or bystander delivery of positive selection signals could potentially lead to the appearance of long-lived self-reactive B cell clones. Cytokines, cytotoxic granules, and more recently neurotransmitters including dopamine were shown to be part of Tfh-B cell interaction and the involvement of a much larger array of neurotransmitter-like molecules is expected to be discovered in the following years (Papa and Vinuesa).

Tfh CELLS IN AUTOIMMUNE DISEASES

Dysregulation of Tfh cell responses has been implicated in various autoimmune and inflammatory disorders in humans and mouse models. Thus, Tfh cell differentiation and maintenance must be closely regulated to ensure appropriate help to B cells (1, 4). Gensous et al. nicely recapitulated the molecular factors involved in Tfh cell formation in the context of a normal immune response, as well as markers associated with their identification (transcription factor, surface marker expression, and cytokine production). In their review article, Petersone et al., emphasized how CTLA-4-mediated regulation of CD28 signaling controls the engagement of secondary costimulatory pathways such as ICOS and OX40, and profoundly influences the T cell/B cell collaboration. Qin et al. summarized the recent findings of Tfh cell frequency and phenotype alteration in autoantibody-mediated diseases, such as systemic lupus erythematosus, Sjögren's syndrome, juvenile dermatomyositis, autoimmune myasthenia gravis, rheumatoid arthritis and type 1 diabetes.

Kim et al. recapitulated the molecular mechanisms for Tfh cell generation, survival and function in both humans and mice, and discussed the relationship between Tfh cells and autoimmune disease in animal models and patients. In their original research article, Felix et al. demonstrated that the P2RX7 receptor, an ATP-gated cation channel whose activation results in the release of pro-inflammatory molecules negatively controlling Tfh cell development in Peyer's patches, is required to control the autoimmune disease by keeping the Tfh cell response at check. Thus, contrary to exerting an anti-inflammatory effect, P2RX7 deficiency enhances autoimmune arthritis (Felix et al.). The results of the interesting clinical study by Cunill et al. demonstrated a pathogenic pro-inflammatory profile of circulating Tfh cells in relapsing-remitting multiple sclerosis patients, defined by high Tfh17/1 and low Tfh2 sub-populations, and the reversion of this biological signature by Dimethyl Fumarate treatment.

Serr and Daniel, in their brief review, reported the state of the art regarding the role of Tfh cells in the development of type 1 diabetes (T1D) and discussed thoroughly advances in the field of Tfh cell differentiation and function during the emergence of human islet autoimmunity. They also presented novel findings on the regulation of Tfh cells by microRNAs

(miRNAs), as well as the potential use of miRNAs as biomarkers to predict disease progression. An analysis of Tfh cell frequencies or miRNAs involved in Tfh cell development in longitudinal samples could help in predicting the progression time to clinically overt T1D (Serr and Daniel). Interestingly, Cosorich et al. provided evidence that Tfh cells expressing gut homing chemokine receptor CCR9 from the gastrointestinal tract exhibit plasticity and, by downmodulating the expression of CXCR5 can migrate to distal accessory organs of the digestive system, like the pancreas, where they may participate in autoimmunity.

An interesting review article about the emerging notion that an IL-1 axis, the key cytokine of innate immune responses predominantly produced by monocytes and macrophages, might control humoral immune responses by Tfh and Tfr cells was provided by Ritvo et al. The discovery of a peculiar distribution of IL-1 receptors and IL-1 antagonists on Tfh and Tfr cells has led to a new understanding of the role of IL-1 in the control of antibody production. Some *in vitro* evidence confirmed a direct role of IL-1 in the activation of Tfh cells and indicated that targeting the IL-1 pathway should be important, although so far ignored, therapeutic approach to many autoimmune diseases, acting not just by reducing inflammation, but also by directly reducing the autoantibody response (Ritvo et al.).

Tfh CELLS IN CHRONIC INFLAMMATION

Lymphocytes migrating into chronically inflamed tissues form ectopic lymphoid structures with functional GCs, also known as tertiary lymphoid structures (TLS). T cells that interact with B cells in these sites, named Tfh-like cells, produce factors associated with B cell help, including IL-21 and the B cell chemoattractant CXCL13, yet vary dramatically in their resemblance to Tfh cells found in secondary lymphoid organs, e.g., surface phenotype, migratory capacity, and transcriptional regulation (10). The review article by Rao discusses observations from multiple diseases and models in which tissue-infiltrating T cells play a significant role in TLS formation. Hutloff also summarizes findings on this topic discovered by studies on experimental animal models as well as some autoimmune and malignant diseases. Both reviews provide an interesting insight into a deeper understanding of these mechanisms in chronically inflamed tissues and suggest approaches to target these cells (Hutloff).

Tfh CELLS IN CANCER

Interesting considerations also for cancer immunology have been generated from the comprehension of the mechanisms of Tfh cell development/maintenance. Accumulating evidence suggests that Tfh cells are involved in peripheral T cell and B cell-associated tumors, for example, in angioimmunoblastic T cell lymphoma (AITL), an aggressive tumor where neoplastic T cells express CXCL13, ICOS, CD154, CD40L, and NFATC1, making these T cells similar to Tfh cells. Follicular T cell lymphomas are another example, where infiltrating T cells resemble Tfh-like cells and

express chemokines that play a role in the regulation of Treg and Th2 cell migration and modulate the activity of GC B cells (10–12). Moreover, the number of Tfr cells was found elevated during the various stages of the lymphoma development (Qin et al.).

On the other side, Tfh cells seem to have protective roles in some non-lymphoid tumors. Higher levels of Tfh cell infiltrates and an elevated presence of TLS within tumors have been associated with increased survival and reduced immunosuppression in patients with breast cancer. Evidence suggests that IL-21 and CXCL13 produced by tumor-infiltrating CD4⁺ T cells may play a critical protective role. Infiltrating Tfh cells have also been reported in chronic lymphocytic leukemia, non-small cell lung cancer, osteosarcoma, and colorectal cancer, where, in some cases, they positively correlated with patient survival (Qin et al.). In their review article Poultsidi et al. raise the question of whether cancer neoantigens can drive Tfh differentiation. Another key question regards Tfh cell homing to lymph nodes and their role in tumor metastasis. Future research will help identify new molecular targets aiming at boosting Tfh cell responses against some types of tumors (Poultsidi et al.).

Tfh CELLS IN INFECTIONS AND VACCINE RESPONSES

CD4⁺ T cell differentiation is influenced by a plethora of intrinsic and extrinsic factors and different classes of pathogens may induce a distinct balance of CD4⁺ T cell differentiation programs (9). Huang et al. recapitulated the molecular basis of virus-specific Tfh cells as part of a process involving multiple factors and stages and exhibiting distinct features. The original research article by Wang et al. demonstrated that the transcription factor T-bet, specifically expressed in type I Tfh cells, was dispensable for the early fate Tfh commitment, but essential for Tfh cell maintenance, proliferation and apoptosis inhibition during acute viral infection. The original research article by Danelli et al. reports an uncommonly strong bias toward Tfh cell differentiation of CD4⁺ T cells reactive with a retroviral envelope glycoprotein model antigen during retroviral infection. The response to the same antigen in different immunization regimens elicited a response typically balanced between Tfh and Th1 cells. Influencing factors for Tfh differentiation were T cell receptor (TCR) signaling that controlled PD-1 expression (Danelli et al.).

Several studies have revealed the important role of Tfh cells in Human Immunodeficiency Virus (HIV) pathogenesis. In the exciting research conducted by McCarty et al. on a Kenyan cohort of 76 perinatally HIV-infected children, HIV treatment-naïve children had reduced levels of cTfh cells compared to healthy children. Memory cTfh cells with elevated PD-1 levels correlated with advancing HIV disease status. Antiretroviral treatment restored cTfh cell frequency but did not decrease PD-1 levels on cTfh cells (Wang et al.). Greczmiel and Oxenius focused their review article on the mechanisms by which Tfh cells induce

neutralizing protective antibody responses toward non- or poorly cytopathic viruses (i.e., HIV-1, HBV, HCV in humans, and LCMV in mice). These humoral responses are fundamental to afford control of the persistent infection—despite the risk of viral escape due to the high mutation rate during virus replication—in the absence of overt immunopathology (Greczmiel and Oxenius).

Tfh CELLS IN PRIMARY IMMUNODEFICIENCIES (PIDs)

Several immunodeficiencies directly affect the development and functions of Tfh cells by impairing GC formation and altering B cell-dependent responses, e.g., mutations in SH2D1A, CD40L, ICOS, and STAT3 (6–8). In their perspective article, Preite et al. describe how PI3K-mediated pathways are likely to integrate multiple signals to promote Tfh cell differentiation, whose dysregulation is mirrored in human PID “Activated PI3K-delta Syndrome” (APDS). An original research article by Klocperk et al. described the number and phenotype of Tfh cells in a cohort of 17 patients with DiGeorge Syndrome, an immunodeficiency characterized by thymic dysplasia with increased susceptibility to infections and autoimmunity. While the population of cTfh cells was significantly expanded in patients with DiGeorge syndrome compared with age-matched healthy controls, their frequency did not significantly differ between DiGeorge patients with or without autoimmune manifestations, allergy, or dysgammaglobulinaemia. The authors concluded that the relative expansion of cTfh cells may be the result of impaired T cell development in patients with thymic dysplasia (Klocperk et al.).

Tfh CELLS IN TRANSPLANTATION TOLERANCE

The role of Tfh cells in transplantation is also a matter of great interest (3). In their original research article, Kwun et al. elucidated the post-transplant B cell immune response after T cell depletion. In a CD52 transgenic mouse model of heterotopic heart transplantation, the use of alemtuzumab, a monoclonal depleting antibody that binds to CD52 expressed on mature lymphocytes, promoted the production of serum donor-specific antibodies, allo-B cells and coronary allograft vasculopathy, a hallmark of chronic rejection. Moreover, hyperplastic GCs with elevated serum IL-21 were detected. The authors observed that the concomitant use of Anti-LFA-1 monoclonal antibody suppressed the humoral response in animals treated with alemtuzumab, providing a novel mechanism and paving the way to possibly new IL-21-directed therapeutic approaches for chronic antibody-mediated rejection (Kwun et al.).

FOLLICULAR REGULATORY T CELLS (Tfr) IN HEALTH AND DISEASE

Tfr cells are a recently identified subset of CD4⁺ FOXP3⁺ T cells that controls humoral immune responses in ectopic follicles and

GCs of secondary lymphoid organs. Recent works have identified the functional and developmental characteristics of Tfr cells and have highlighted their characteristics of differentiation, GC recruitment and retention, and regulatory abilities. Moreover, Tfr cells finely regulate the balance of pathogen-specific to autoantibody production by constantly interacting with Tfh and B cell populations and altering their environment through cytokine production and sequestration, thereby influencing the quantity and quality of the GC response (1, 2).

In their review article, Fazilleau et al. focused on the role of Tfr cells as “negative regulators” dedicated to control the magnitude of the immune response in the GC, and thoroughly described the Tfr cell proprieties in the context of vaccination. On the same line, in their review, Miles and Connick summarize the current knowledge about Tfr cells in response to infection and their potential role in vaccine development. In the review article by Wing et al. the role of Tfr cells and the contribution activated extra-follicular Tregs (eTreg) in the control of humoral immunity, as well as the role of Tfr cells in autoimmune diseases and tumors, is summarized.

In the review article by Stebegg et al., an insightful overview of the complex and multilevel regulation of the GC is provided, including the biology of stromal cell subsets and chemokines network in both secondary lymphoid tissues and Peyer’s patches. Xie et al. review article is focused on Tfr cell functions and discuss the evidence that Tfr cells can also play a major “helper” role in the GC-dependent antibody response by producing IL-10 that promotes GC B cell growth and high-affinity antibody production. Thus, in the context of the GC response, Tfr cells appear to maintain a key balance between help (GC maintenance, antibody response, and affinity) and suppression by controlling Tfh cell numbers, GC B cell numbers, Tfh cell cytokines, and autoantibodies (Xie et al.).

CONCLUSIONS

Despite all the progress made in the last three decades, we are still at an early stage in our understanding of the sophisticated and multi-level role of Tfh and Tfr cells in health and disease. The complex niche of the GC is governed by delicate cognate interactions between Tfh, Tfr, B cells and stromal cells, the role and potential of the latter still need to be fully clarified. Evidence indicates that most patients affected by autoimmune diseases have increased numbers of Tfh cells that are also hyperactive, and possess altered numbers of Tfr cells with reduced function. Great interest is emerging on the role of Tfh and Tfr cells in PID and transplantation, where further studies may lead to the discovery of new therapeutic strategies and biological paradigms. Novel insights are also emerging on the role of Tfh and Tfr cells in tumors, allergy, infections, and vaccine responses that, together with the comprehension of the molecular mechanisms underlying the development and function of Tfh and Tfr cells in these clinical settings, may lead to the discovery of novel therapeutic targets. Increased knowledge of Tfh cells and Tfr cells has inspired, and hopefully

it will continue to inspire more studies to reinstate the balance of these cells for the prevention and treatment of diverse human diseases.

AUTHOR CONTRIBUTIONS

MC, SS-A, and GF have made a substantial, direct and intellectual contribution to the writing of this editorial, and approved it for publication.

REFERENCES

- Vinuesa CG, Linterman MA, Yu D, MacLennan IC. Follicular helper T cells. *Annu Rev Immunol.* (2016) 34:335–68. doi: 10.1146/annurev-immunol-041015-055605
- Fonseca VR, Graca L. An overview of T follicular cells in transplantation: spotlight on their clinical significance. *Clin Exp Immunol.* (2019) 195:302–4. doi: 10.1111/cei.13245
- Niu Q, Kraaijeveld R, Li Y, Mendoza Rojas A, Shi Y, Wang L, et al. T follicular helper cells and T follicular regulatory cells in rheumatic diseases. *Expert Rev Clin Immunol.* (2019) 15:1249–62. doi: 10.1080/1744666X.2020.1693262
- Deng J, Wei Y, Fonseca VR, Graca L, Yu D. T follicular helper cells and T follicular regulatory cells in rheumatic diseases. *Nat Rev Rheumatol.* (2019) 15:475–90. doi: 10.1038/s41584-019-0254-2
- Vecchione A, Gerosa J, Di Fonte R, Jofra T, Cicalese MP, Napoleone V, et al. Reduced PD-1 expression on circulating follicular and conventional FOXP3⁺ Treg cells in children with new onset type 1 diabetes and autoantibody-positive at-risk children. *Clin Immunol.* (2019) 30:108319. doi: 10.1016/j.clim.2019.108319
- Gerosa J, Lougaris V, Baronio M, Plebani A, Cicalese MP, Foustieri G. Beta2 integrins are required for follicular helper T cell differentiation in humans. *Clin Immunol.* (2017) 180:60–2. doi: 10.1016/j.clim.2017.04.002
- Cicalese MP, Gerosa J, Baronio M, Montin D, Licciardi F, Soresina A, et al. Circulating follicular helper and follicular regulatory T cells are severely compromised in human CD40 deficiency: a case report. *Front Immunol.* (2018) 9:1761. doi: 10.3389/fimmu.2018.01761
- De Leo P, Gazzarelli L, Baronio M, Montin D, Di Cesare S, Giancotta C, et al. NFKB2 regulates human Tfh and Tfr pool formation and germinal center potential. *Clin Immunol.* (2019) 210:108309. doi: 10.1016/j.clim.2019.108309
- Zhang J, Liu W, Wen B, Xie T, Tang P, Hu Y, et al. Circulating CXCR3⁺ Tfh cells positively correlate with neutralizing antibody responses in HCV-infected patients. *Sci Rep.* (2019) 9:10090. doi: 10.1038/s41598-019-46533-w
- Couillault C, Germain C, Dubois B, Kaplon H. Identification of tertiary lymphoid structure-associated follicular helper T cells in human tumors and tissues. *Methods Mol Biol.* (2018) 1845:205–22. doi: 10.1007/978-1-4939-8709-2_12
- Lemonnier F, Mak TW. Angioimmunoblastic T-cell lymphoma: more than a disease of T follicular helper cells. *J Pathol.* (2017) 242:387–90. doi: 10.1002/path.4920
- Gu-Trantien C, Migliori E, Buisseret L, de Wind A, Brohée S, Garaud S, et al. CXCL13-producing TFH cells link immune suppression and adaptive memory in human breast cancer. *JCI Insight.* (2017) 2:e91487. doi: 10.1172/jci.insight.91487
- Li J, Lu E, Yi T, Cyster JG. EBI2 augments Tfh cell fate by promoting interaction with IL-2-quenching dendritic cells. *Nature.* (2016) 533:110–4. doi: 10.1038/nature17947

FUNDING

We thank the support of the San Raffaele Hospital (Ospedale San Raffaele) (5x1000 OSR PILOT & SEED GRANT) to GF and MC. SS-A was supported with NIH grants AI77079 and AI087734.

ACKNOWLEDGMENTS

We want to sincerely thank all of the authors who contributed to this collection of articles.

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2020 Cicalese, Salek-Ardakani and Foustieri. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



The Janus Face of Follicular T Helper Cells in Chronic Viral Infections

Ute Greczmiel and Annette Oxenius*

Institute of Microbiology, ETH Zürich, Zürich, Switzerland

OPEN ACCESS

Edited by:

Georgia Fousteri,
San Raffaele Hospital (IRCCS), Italy

Reviewed by:

Ramon Arens,
Leiden University Medical Center,
Netherlands
Karl Lang,
University of Essen,
Germany

*Correspondence:

Annette Oxenius
oxenius@micro.biol.ethz.ch

Specialty section:

This article was submitted to
T Cell Biology,
a section of the journal
Frontiers in Immunology

Received: 06 February 2018

Accepted: 09 May 2018

Published: 25 May 2018

Citation:

Greczmiel U and Oxenius A (2018)
The Janus Face of Follicular T Helper
Cells in Chronic Viral Infections.
Front. Immunol. 9:1162.
doi: 10.3389/fimmu.2018.01162

Chronic infections with non-cytopathic viruses constitutively expose virus-specific adaptive immune cells to cognate antigen, requiring their numeric and functional adaptation. Virus-specific CD8 T cells are compromised by various means in their effector functions, collectively termed T cell exhaustion. Alike CD8 T cells, virus-specific CD4 Th1 cell responses are gradually downregulated but instead, follicular T helper (T_{FH}) cell differentiation and maintenance is strongly promoted during chronic infection. Thereby, the immune system promotes antibody responses, which bear less immune-pathological risk compared to cytotoxic and pro-inflammatory T cell responses. This emphasis on T_{FH} cells contributes to tolerance of the chronic infection and is pivotal for the continued maturation and adaptation of the antibody response, leading eventually to the emergence of virus-neutralizing antibodies, which possess the potential to control the established chronic infection. However, sustained high levels of T_{FH} cells can also result in a less stringent B cell selection process in active germinal center reactions, leading to the activation of virus-unspecific B cells, including self-reactive B cells, and to hypergammaglobulinemia. This dispersal of B cell help comes at the expense of a stringently selected virus-specific antibody response, thereby contributing to its delayed maturation. Here, we discuss these opposing facets of T_{FH} cells in chronic viral infections.

Keywords: follicular helper cells, chronic viral infection, antibody responses, germinal center, viral evolution

INTRODUCTION

Non-or poorly cytopathic viruses like human immunodeficiency virus 1 (HIV-1), hepatitis B virus (HBV), and hepatitis C virus (HCV) in humans or lymphocytic choriomeningitis virus (LCMV) in mice can induce persistent infections employing several mechanisms to evade control by the immune system. Continuous high-level viral replication and therefore high viral burden in the host is a major factor leading to numeric reduction and functional impairment of virus-specific cytotoxic CD8 T cells and Th1 CD4 T cells, collectively termed T cell exhaustion [reviewed in Ref. (1–3)]. In this setting, immune effector functions being less prone to induce immunopathology, like the humoral arm of immunity, are beneficial to contain viral spread (4–10). Especially, virus-neutralizing antibodies can inhibit new infection of host cells and thereby effectively limit viral spread. However, isotype-switched neutralizing antibodies often appear very late after the onset of persistent viral infections, being frequently delayed for several weeks to months (5, 11, 12). B cell dysregulation, including hypergammaglobulinemia and polyclonal B cell activation, contributes to the late emergence of virus-neutralizing antibodies (13, 14). Furthermore, mutational viral evolution results in selection of variants that escape the neutralizing antibody response, promoting persistence of the infection (12, 15–22).

TABLE 1 | Regulation and role of T_{FH} cells in chronic viral infections.

Follicular T helper (T _{FH}) cells	Impact	Consequence	Reference	
Lymphocytic choriomeningitis virus (LCMV) infection				
Sustained T _{FH} activity	Generation of LCMV-neutralizing antibodies	Eventual control of infection	(7)	Positive role of T _{FH} for antibody responses and eventual virus control
Lack of T _{FH} cells from the onset of infection	Impaired antibody response	Sustained persistent infection	(8, 10, 38, 86–88)	
Increase of T _{FH} cells by NK cell depletion	Improved antibody response	Enhanced/accelerated virus control	(89)	
Sustained high levels of T cell help	Polyclonal B cell activation (including autoreactive B cells)	Hypergammaglobulinemia	(13, 202)	Negative impact of sustained T _{FH} responses
Simian immunodeficiency virus (SIV)/HIV/hepatitis C virus (HCV)/hepatitis B virus (HBV) infection				
High frequencies of T _{FH} cells in SIV infection	Correlation with high-affinity SIV-specific antibodies		(43, 90)	Positive role of T _{FH} for antibody responses
Reduction of follicular regulatory T (T _{FR}) cells in SIV infection	Increased T _{FH} cell numbers	High avidity antibodies to SIV gp120	(96, 126)	
High frequencies of (functional) (c)T _{FH} cells in HIV, HCV, and HBV infection	Broad (neutralizing) antibody responses	Positive correlation with antibody affinity	(42, 91–95, 97, 98)	
T _{FH} in HCV infection	Reduced IL-21 production	Normal <i>in vitro</i> help to B cells	(124)	Negative impact of sustained T _{FH}
Reduced T _{FH} frequencies in spleen in SIV infection	Reduced SIV-specific IgG titers		(107)	
Loss of T _{FH} cells in advanced chronic SIV infection	Impairment of B cell response	Progression to AIDS	(100)	
cT _{FH} with impaired function in HIV infection	No correlation with neutralizing antibodies	Impaired function	(95, 99)	
Reduced T _{FH} function in HIV infection	Caused by PD ligand 1 (PD-L1) expression on B cells	Blockade of PD-L1 restores T _{FH} function	(123)	
Increased T _{FR} response in SIV and HIV infection	Insufficient germinal center response		(127)	
Increased T _{reg} and B _{reg} in HCV and HBV infection	Impaired antibody response?		(108, 130–132)	
Direct infection of T _{FH} by HIV/SIV	Impaired function	Viral reservoirs	(135–137)	
Sustained high levels of T cell help in SIV, HIV, HCV, and HBV infection	Polyclonal B cell activation s (including autoreactive B cells)	Hypergammaglobulinemia	(42, 43, 199, 200, 203–205)	

Green: positive role of TFH cells in chronic infections. Orange: negative role of TFH cells in chronic infections. White: unassigned role of TFH cells in chronic infection.

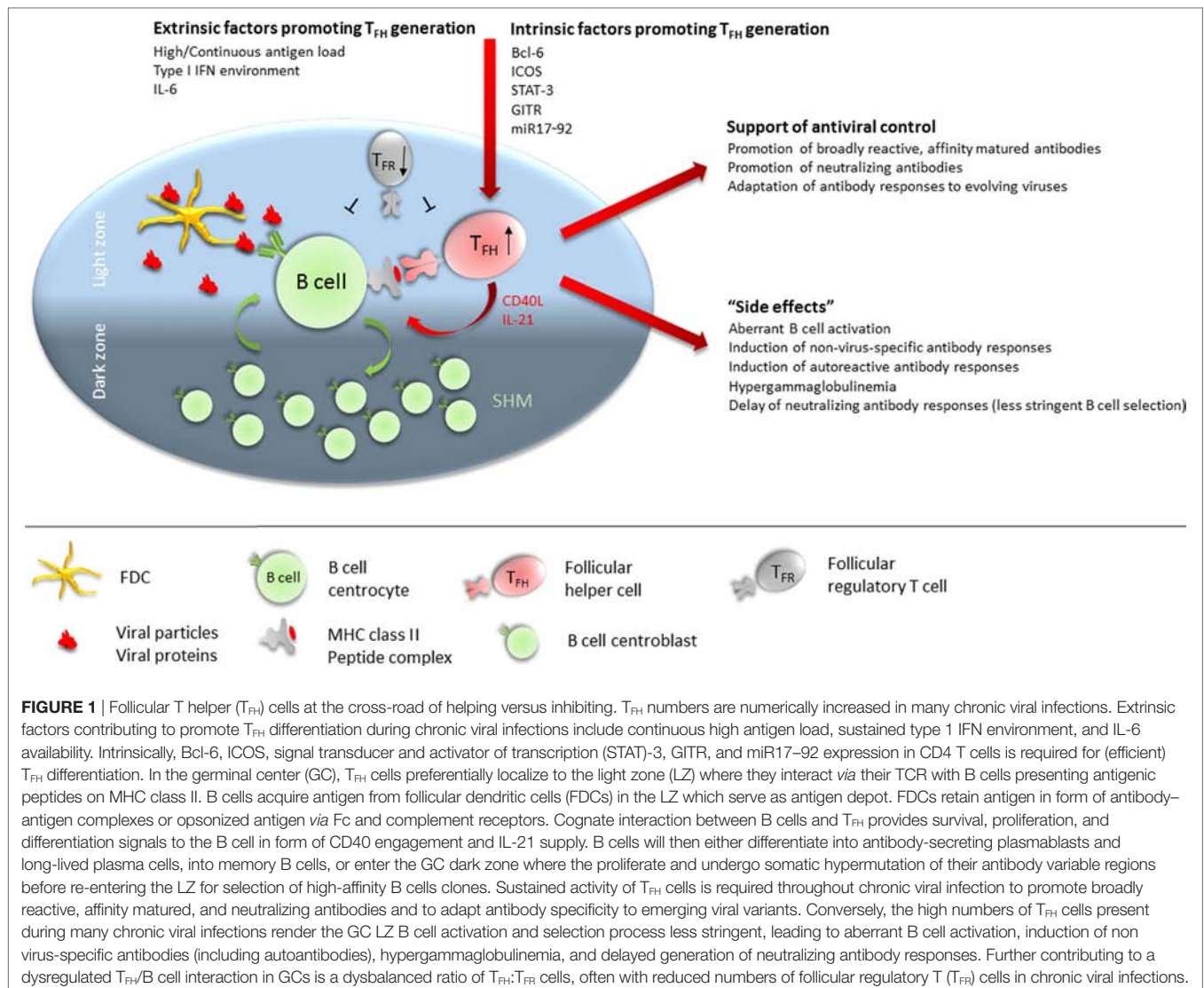
Isotype-switched antibody responses are elicited in a T-help-dependent manner, being regulated by the interaction between follicular T helper (T_{FH}) cells and cognate B cells (23). Activity of T_{FH} cells is regulated by the transcriptional repressor B cell lymphoma (Bcl)-6 (24–26) which sustains, among other functions, upregulation of the chemokine receptor CX-chemokine receptor (CXCR) 5 that in turn mediates localization of T_{FH} cells to the B cell follicle (27–29). There, T_{FH} cells initiate B cell differentiation into either short-lived plasmablasts or germinal center (GC) B cells (30–33). Conversely, contact between T_{FH} cells and cognate B cells is necessary to induce differentiation of T_{FH} cells into GC T_{FH} cells and to sustain their T_{FH} phenotype (34–37), albeit this is disputed to also hold in case of persistent viral infections (38). T_{FH} cells mediate affinity selection of B cells that have undergone proliferation and somatic hypermutation (SHM) by delivering survival signals *via* ICOS, CD40 ligand (CD40L), and the cytokine IL-21, depending on the affinity of the B cell for a given antigen (39–41). Therefore, T_{FH} cells are essential for the induction and maintenance of the GC response.

Interestingly, T_{FH} cells accumulate during the persistent phase of viral infections with non- or poorly cytopathic viruses (8, 38, 42, 43) while differentiation of naïve CD4 T cells into Th1 CD4 T cells is largely abrogated in this phase due to a sustained IFN-I environment (44). The expansion of the T_{FH} population is most likely

driven by follicular dendritic cell (FDC)-derived IL-6 signaling *via* signal transducer and activator of transcription (STAT)-3 (8, 43, 45), and the prolonged persistence of viral antigen in the host environment (46). It would be intriguing to conjecture an essential role of the sustained expansion of the T_{FH} cell population for the eventual induction of the virus-neutralizing antibody response and also adaptation of the protective response to an evolving virus. However, accumulation of T_{FH} cells might also contribute to the observed B cell dysregulation and thereby delay of the neutralizing antibody response (**Figure 1**). Here, we discuss evidence for both, promotion of late emergence of virus-neutralizing antibodies and dysregulated B cell responses in the context of chronic viral infections, focusing on experimental LCMV infection in mice and HIV-1, HCV, and HBV infection in humans (**Table 1**).

T_{FH} CELLS

Follicular T helper cells are the main regulators of T-help-dependent antibody responses (23). Instruction of T_{FH} cell differentiation is mediated in two steps. Priming of CD4 T cells that commit to the T_{FH} cell lineage takes place in the T cell zone and is mediated by conventional DCs or monocyte-derived DCs (47, 48). In a second step, differentiation to T_{FH} cells is further instructed and the T_{FH} phenotype stably established by



interactions between primed T_{FH} cells and B cells at the border between T cell and B cell zone.

Which factors/cytokines instruct T_{FH} differentiation is not entirely resolved, but both IL-6 and IL-21 can induce T_{FH} differentiation *via* signaling through the transcription factor STAT-3 (49, 50). In the context of a persistent LCMV infection, it has furthermore been shown that late FDC-derived IL-6 is essential for T_{FH} cell maintenance and eventual control of the infection (8).

CD4 T cells differentiating to T_{FH} cells upregulate the hallmark transcriptional repressor Bcl-6 (24–26). Bcl-6 promotes commitment to the T_{FH} cell lineage by repression of Blimp-1, which mediates expression of genes that are involved in the differentiation into other CD4 T cell lineages (24). Furthermore, Bcl-6 promotes localization of T_{FH} cells toward the B cell follicle where T-help-dependent antibody responses take place. This is achieved in two different ways. For one, Bcl-6 represses the expression of molecules promoting localization in the T cell zone or egress from secondary lymphoid organs, i.e., CC chemokine receptor-7, Epstein–Barr virus-induced G-protein-coupled receptor (EBI)-2,

or P-selectin glycoprotein-1 (51–53). Furthermore, Bcl-6 stabilizes the expression of CXCR5 on T_{FH} cells, which is upregulated by the transcription factor achaete-scute homolog-2 (ASCL-2) upon priming (54). CXCR5 is essential for the localization of T_{FH} cells toward the CXCL13-rich B cell follicles (27–29). T_{FH} cells can further be distinguished by expression of other typical markers, which have important functions in mediating cognate interactions with B cells and thus sustaining antibody responses. Among these markers are the costimulatory molecules inducible T-cell costimulator (ICOS) and CD40L, the immunoregulatory molecule PD-1, their hallmark cytokine IL-21, and the T cell adaptor protein SAP (23, 55–58). Expression of these markers is moderate after priming and needs to be sustained and increased by interaction of T_{FH} cells with cognate B cells and by ICOS signals delivered by ICOS ligand expressing bystander B cells in the interfollicular zone (34–37). These first interactions between T_{FH} cells and B cells also determine the differentiation of T_{FH} cells into GC T_{FH} cells that induce and maintain the GC response and play an important role in the positive selection of affinity

matured B cell clones. Expression of T_{FH} markers is highest in GC T_{FH} cells (37, 59, 60). After an immune response, some T_{FH} cells have been shown to differentiate into long-lived memory cells, which downregulate some of their typical T_{FH} markers like CXCR5, Bcl-6, and PD-1 (61, 62).

In humans, there have been further reports about circulating T_{FH}-like cells that express CXCR5 and display a memory phenotype. Their expression of ICOS, PD-1, and Bcl-6 is reduced as well. However, these cells are efficient producers of IL-21 and IL-10 in *in vitro* coculture and effective inducers of B cell differentiation (23).

Closely related to T_{FH} cells and equally important for the regulation of the GC responses are the so-called follicular regulatory T (T_{FR}) cells. These are derived from thymus-derived T regulatory (T_{reg}) cells, which adopt some T_{FH} cell characteristics, like CXCR5 and Bcl-6 expression, to be able to migrate into B cell follicles. However, T_{FR} cells lack expression of CD40L, IL-4, or IL-21 and have a higher expression of PD-1 and negatively regulate the GC response (63–65).

GC RESPONSE

The first encounter between T_{FH} cells and activated B cells occurs in the interfollicular zone which lies at the border between T cell zone and B cell follicle (66–68). Here, interaction between T_{FH} cells and cognate B cells induces a first round of B cell proliferation and instructs them to undergo one of three possible differentiation pathways. Either B cells undergo differentiation into short-lived extra-follicular plasmablasts, which produce a first wave of low affinity antibodies, or into GC-independent memory B cells or into GC B cells (30–33, 69). B cells destined to induce the GC response migrate with a subset of T_{FH} cells, GC T_{FH} cells, further into the B cell follicle. This migration is mediated by downregulation of EBI2 and upregulation of Sphingosine-1-phosphate receptor 2 on both B and T_{FH} cells (66, 69–71).

The GC is partitioned into two distinct zones, the dark zone (DZ) and the light zone (LZ). In the DZ the cytokine CXCL12 is predominantly produced while the cytokine CXCL13 is predominantly produced in the LZ. Thereby, localization of B cells in DZ and LZ is controlled by differential expression of the chemokine receptors CXCR4 (migration into DZ) and CXCR5 (migration into LZ) (72). In the DZ, B cells undergo sequential rounds of proliferation (73–76). During this process, B cells upregulate the activation-induced cytidine deaminase (AID) which introduces point mutations into the variable regions of the B cell expressed BCRs/antibodies, a process termed SHM (77–79). Thereby, clonal B cell variants with different affinities toward one given antigen are generated. The activity of AID is also essential for class-switch reactions, which change the isotype of the antibodies (77, 79).

As SHM is a random process, it is necessary for B cells to undergo a selection process to ensure affinity maturation of the antibody repertoire and to exclude B cells that lost affinity for one antigen, decreased their affinity or even developed into autoreactive B cells. This selection process takes place in the LZ of the GC where also most of the GC T_{FH} cells and FDCs are located (73, 75, 76). Upon entry into the LZ, B cells take up antigen which is stored on/presented by FDCs *via* their mutated BCR according

to their affinity toward the antigen. Afterward, B cells present processed antigen to cognate GC T_{FH} cells *via* their surface MHC II molecules. Higher affinity B cells are believed to have a competitive advantage in taking up FDC-stored antigen and thus are able to present more antigen on their surface MHC II molecules (80). The amount of presented antigen determines the amount of survival signals *via* ICOS, CD40L, and IL-21 a B cell clone receives from cognate GC T_{FH} cells (39, 40, 76).

B cell clones that do not receive sufficient survival signals and therefore are negatively selected undergo apoptosis mediated by binding of Fas, expressed by the B cell, to FasL, expressed by the GC T_{FH} cell (41, 81). Positively selected B cell clones either undergo another round of proliferation and SHM in the DZ or leave the GC reaction as long-lived plasma cells or memory B cells (74, 75, 82). B cells with the highest affinity may preferentially differentiate into plasma cells (69, 83, 84). B cell clones with a lower affinity, however, rather differentiate into memory B cells (82).

T_{FH} CELLS ARE ESSENTIAL FOR THE EMERGENCE OF VIRUS-NEUTRALIZING ANTIBODIES AND CONTROL OF PERSISTENT VIRAL INFECTION

The role of T_{FH} cells for viral control during persistent viral infections, which is assumed to be dependent on development of neutralizing antibodies during the GC response (13, 16, 85), has been widely studied in the setting of persistent LCMV infection. For example, mice harboring a constitutive CXCR5 deficiency, and therefore being unable to develop T_{FH} cells (and B cell follicles), exhibit an abrogated antibody response and prolonged viral persistence (38). Likewise, IL-6^{-/-} (8), IL-6 signaling-deficient (10), STAT3^{-/-} (86), glucocorticoid-induced tumor necrosis factor receptor related protein (GITR)-deficient mice (87), and mice with a T cell-specific deletion of the miR17–92 family of microRNAs (88) fail to elicit or maintain a T_{FH} cell response upon (persistent) LCMV infection and are unable to eventually control the infection. Conversely, increasing the number of T_{FH} cells by NK cell depletion accelerated viral clearance by improving the virus-specific antibody response (89).

Similar correlations between T_{FH} cells and the appearance of protective antibody responses were observed in other persistent viral infections, e.g., with simian immunodeficiency virus (SIV), where the frequency of T_{FH} cells positively correlated with the appearance of high-affinity SIV-specific antibodies in infected rhesus macaques (RM) (43). These T_{FH} cells adopted a Th1-like profile regarding their chemokine receptor and cytokine expression (90). Furthermore, the quantity of T_{FH} cells was higher in slow/non-progressor SIV-infected RMs, in which the virus was better contained, as compared with progressor SIV-infected RMs. The increase in T_{FH} cell numbers in slow progressor correlated with higher titers of SIV-specific IgG antibodies in serum of infected RMs (43). Also, in chronically infected HIV-1, HCV and HBV patients, increased frequencies of a circulating population of cells with T_{FH} characteristics (cT_{FH}) (CXCR5⁺CXCR3⁺PD-1⁺) were observed (42, 91–98), which seemed closely related to GC T_{FH} cells, based on their gene expression and cytokine profile. These

were able to induce B cell differentiation *in vitro* and correlated with the appearance of broadly HIV-neutralizing antibodies (91, 92). In HIV controllers, an expanded population of functional gp120-specific T_{FH} cells in blood correlated with gp120-specific B cell frequencies (93). However, other studies reported a reduced capacity of cT_{FH} cells to provide help to B cells in (advanced) HIV-1 infected individuals (95, 99) or even loss of T_{FH} cells in SIV-infected RMs (100).

Another indication implicating T_{FH} cells in the eventual emergence of virus-neutralizing antibodies during persistent viral infection is the high frequency of somatic mutations in the variable regions of these antibodies (11, 19, 101, 102). SHM predominantly takes place in the GC and selection of high-affinity clones is supported by GC T_{FH} cells (76). It is therefore tempting to speculate that continued activity of T_{FH} cells during chronic viral infections is required for a continuous selection process of virus-specific B cell clones. This results not only in a continuous increase of their affinity toward viral antigens but also allows them to evolve to bind (and neutralize) to viral quasi species that emerge *in vivo* under selection pressures.

Indeed, we recently presented experimental evidence that sustained presence of CXCR5^{+/+} or Bcl6^{+/+} T_{FH} cells is strictly required for the (late) emergence of LCMV-neutralizing antibodies. Using a novel *in vivo* experimental system allowed conditional depletion of specifically T_{FH} cells or all LCMV-specific CD4 T cells during established persistent LCMV infection, after the initial establishment of the virus-specific IgG antibody response (7). This permitted, in contrast to previous studies (8, 10, 38, 86), to examine the function of T_{FH} cells and LCMV-specific CD4 T cells during persistent viral infection beyond the mere induction of the virus-specific antibody response. This study revealed that LCMV-specific T_{FH} cells (i.e., CXCR5^{+/+} or Bcl6^{+/+} CD4 T cells) were dispensable for maintaining overall LCMV-specific IgG titers and LCMV-specific IgG secreting plasma cells in spleen and bone marrow. By contrast, continued presence of LCMV-specific CD4 T cells was required to maintain overall LCMV-specific IgG titers as well as LCMV-specific IgG secreting plasma cells in bone marrow, suggesting that non-T_{FH} LCMV-specific CD4 T cells are able to support an extra-follicular response to maintain the pool of LCMV-specific antibody-secreting cells and hence LCMV-specific IgG titers. However, sustained activity of T_{FH} cells was strictly required for the development of LCMV-neutralizing antibodies by GC B cells (7), as conditional depletion of T_{FH} cells reduced GC B cell numbers and abrogated emergence of antibodies with neutralizing capacity. Moreover, T_{FH} cells seemed to be essential in driving the adaptation of the IgG response toward the contemporaneous circulating LCMV species, lending support to the notion that sustained T_{FH} activity is important for continued selection of B cells. Importantly, the appearance of neutralizing antibodies was required for eventual control of an established persistent LCMV infection, demonstrating the importance of these antibodies and sustained presence and activity of T_{FH} cells for control of a persistent infection in absence of overt immunopathology (7).

Nevertheless, the belated appearance of neutralizing antibodies in the setting of such persistent infections indicates possible restrictions of T_{FH} cell function and/or their interactions with cognate B cells, which are discussed in the following sections.

FACTORS CURTAILING T_{FH} CELL FUNCTION UPON PERSISTENT VIRAL INFECTION

Optimal delivery of T_{FH} cell help to cognate B cells as well as optimal T_{FH} cell differentiation includes a chain of distinct steps at specific localizations in lymphoid tissue as well as a series of cell–cell interactions (23). With respect to localization-dependent processes, the structural integrity of secondary lymphoid organs is crucial allowing for initial encounter of activated CD4 and B cells as well as the establishment of GCs in the B cell follicle, including dark and LZ as designated compartments for proliferation, SHM and B cell selection. Cell–cell interactions that support B cell activation and production of (affinity-matured) antibodies comprise direct contact between activated CD4 T cell and cognate B cells initially at the T/B border, and later between T_{FH} cells and cognate B cells in the GC LZ. Interference with any of these steps might lead to suboptimal antibody responses, which negatively affects control of persistent viral infections.

Destruction of Lymphoid Architecture

One possible influence on the establishment and the quality of T_{FH} and GC B cell responses upon persistent viral infection is (immune-mediated) destruction of the lymphoid tissue architecture (11, 103–110). In chronic LCMV infection, this destruction is largely due to CD8 T cell-mediated cytotoxic activity directed against infected stromal cells as well as sustained type 1 IFN signaling and has been shown to hamper cognate interactions between T and B cells (11, 103–105, 111, 112). In SIV or HIV infection, immune activation-induced fibrosis of lymphoid tissues seems to play a major role in functional deterioration of secondary lymphoid organ structure and function, mediated by T_{reg}-dependent transforming growth factor-beta 1 signaling and ensuing collagen deposition (109, 110).

Simian immunodeficiency virus-infected RMs with an expanded T_{FH} cell population and increased SIV-specific antibody responses displayed a more intact lymph node (LN) structure as compared with fast progressing SIV-infected RMs with a less expanded T_{FH} cell compartment (43, 107). This indicates that an intact lymphoid architecture is beneficial for virus-specific antibody responses and containment of the persistent infection.

Although destruction of lymphoid organ architecture is often attributed to cytotoxic CD8 T cells (104), an additional involvement of cytotoxic CD4 T cells during persistent LCMV infection has been shown (113). Cytotoxic CD4 T cells specifically targeted marginal zone (MZ) B cells, MZ macrophages, and metallophilic macrophages (113), subsets which have been implicated in the optimal induction of antibody responses (114–116). Analogous, depletion of MZ B cells was also reported in the context of persistent HIV infection (117) and a strong T helper response, possibly comprising cytotoxic CD4 T cells, is associated with low neutralizing antibody titers in persistent HCV infection (118).

During persistent LCMV infection, restoration of the lymphoid tissue architecture is closely associated with the onset of the neutralizing antibody response occurring between d40 and d80 post-infection (pi) (5, 103). During acute LCMV infection,

lymphoid architecture is disrupted by day 8 pi and full reorganization, initiated by viral clearance and contraction of the CD8 T cell response, is only completed by d25 pi (103). During persistent LCMV infection, due to persistence of viral antigen and prolonged activity of CD8 and CD4 T cells before undergoing T cell exhaustion (1, 119–121), disruption of the lymphoid organ architecture is likely protracted as compared with acute LCMV infection. This underscores the relevance of lymphoid tissue reorganization and the onset of the LCMV-neutralizing antibody response, further emphasizing the beneficial effect of an intact lymphoid architecture and thereby optimal T and B cell interactions for the development of virus-neutralizing antibodies.

B Cell Dysfunction

In the context of HIV and SIV infection, B cell dysfunction was reported by a number of studies, characterized by loss of naïve and resting memory B cells, increases of activated B cells and tissue-like memory B cells, expansion of regulatory B cells, and altered functionality [reviewed in Ref. (122)].

In SIV or HIV infection, B cells were reported to actively render T_{FH} cells ineffective in delivering help to B cells. GC B cells isolated from HIV or SIV-infected individuals/animals displayed a higher expression of PD ligand 1 (PD-L1) as compared with B cells isolated from healthy donors. Therefore, T_{FH} cells received more signals *via* PD-1 during HIV/SIV infections, which mediated downregulation of IL-21 and IL-4 expression, and at the same time had a negative impact on T_{FH} cell survival and proliferation (123). This impaired their B helper capacity, as observed in *in vitro* coculture experiments. Blocking of PD-L1 on B cells derived from HIV or SIV-infected donors, however, increased the ability of T_{FH} cells to provide help to B cells as well as their cytokine expression (123). This also proved that T_{FH} cells are in principle capable of providing sufficient help to B cells.

Furthermore, cT_{FH} cells exhibiting reduced IL-21 expression as compared with healthy donors were identified in blood of persistently HCV infected patients (124). Surprisingly, however, in contrast to HIV and SIV infection, these cells proved to be capable of providing help to B cells in *in vitro* coculture experiments (124). These differences might be due to the different usage of B cell subsets in the coculture settings. Cocultures in the context of HIV/SIV infection were set up with GC-enriched B cells (123) while cocultures in the context of HCV infection used memory B cells (124). It is also conceivable that different non-or poorly cytopathic viruses use different mechanisms to render the antibody response ineffective upon persistent infection.

Altered Ratios of Regulatory Cells

In the setting of a recent HIV vaccination trial, it was established that the ratio of T_{FH} cells to GC B cells is more important for the quality of the antibody response and eventual emergence of neutralizing antibodies than the total cell numbers. In this context, interaction of few GC B cells with one T_{FH} cell was positively correlated with the occurrence of broadly neutralizing antibodies (bnab) (125). Furthermore, GC responses are subject to regulation by regulatory cells, in particular by T_{FR} cells, which control the GC response to prevent aberrant production of antibodies (64). It has been shown in some studies that the frequency of

T_{FR} cells is reduced upon persistent infection with HIV and SIV (96, 126), albeit other studies have reported an increase of this population in HIV and SIV infection (127). Decreased levels of T_{FR} cells favor the observed expansion of T_{FH} cells and could indicate a less regulated GC response, hampering the induction of protective antibody responses for instance by a less stringent selection process and promoting unspecific B cell activation leading to hypergammaglobulinemia. Conversely, an expanded T_{FR} population might contribute to inefficient GC responses [reviewed in Ref. (128)].

Also, in the context of persistent LCMV infection of lymphopenic mice lacking regulatory T cells, the induction of protective antibody responses was shown to be impaired (46, 129). Adoptive transfer of T_{reg} improved the LCMV-specific antibody response and viral clearance drastically (129), proving the importance of balanced ratios between regulatory cells and T_{FH} and GC B cells during the GC reaction.

Interestingly, in contrast to SIV, HIV and LCMV infection in lymphopenic mice, persistently HCV- or HBV-infected patients displayed an increase of regulatory B cells and T_{regs} as compared with healthy donors. This was associated with increases in IL-10 expression and increased PD-L1 expression on T_{reg} cells (108, 130–132), which together might impair HCV- and HBV-specific antibody responses being associated with poor virus elimination and damage to lymphoid tissue (108).

Accumulation of T_{FH} Cells Which Are Not Specific for Antigens Carrying Neutralizing Epitopes

Upon HIV infection, a predominant expansion of T_{FH} cells that are specific for group-specific antigen is reported (42). However, induction of bnab seems to be associated with Env-specific T_{FH} cells (91). Therefore, specific expansion of T_{FH} cell populations, which are not recognizing the protein carrying neutralizing epitopes, could further contribute to the delayed emergence of neutralizing antibodies. T_{FH} cells with other specificities would predominantly favor the survival of B cells expressing antibodies that are not specific for the neutralizing epitope. However, such intramolecular T cell help does not seem to be generally required and depends on the structure of the B cell activating viral antigen. While individual viral proteins engaging specific BCR would require intramolecular help, B cells interacting with intact or defective virions or virus-derived protein complexes could also be activated by T_{FH} cells that are not necessarily specific for the protein containing the neutralizing epitopes (133, 134). Thus, it would be interesting to understand in more detail the structures of the selecting viral antigens/antigen complexes in the context of persistent viral infections to delineate more precisely the specificities of beneficial T_{FH} responses.

Direct Infection of T_{FH} and T_{FR} Cells in HIV and SIV Infection

Upon HIV and SIV infection, T_{FH} functionality is additionally compromised by their direct infection. CXCR5⁺ CD4 T cells are generally more permissive for HIV and SIV infection as compared with CXCR5[−] CD4 T cells, with GC T_{FH} exhibiting the highest

permissiveness (135–137). T_{FR} cells are also highly permissive for HIV infection—even more so than T_{FH} cells (138).

Surprisingly, infected T_{FH} cells are not directly eliminated as compared with infected CXCR5⁺ CD4 T cells. This might be due to the fact that only few CD8 T cells express CXCR5 and therefore cannot efficiently enter the B cell follicle where the infected T_{FH} cells reside (136, 139, 140). In that way, T_{FH} cells serve as viral reservoirs. However, during SIV and HIV infection, the CXCR5⁺ CD8 T cells that enter the GCs seem to contribute to control of infection (141), or alternatively negatively regulate T and B cells responses *via* IL-10 and Tim3-dependent processes (142). Infected GC T_{FH} cells downregulate T_{FH} markers during active viral replication (135) which might negatively affect their B cell helper functions, rendering the induction of antibody responses less effective.

VIRAL EVOLUTION CAN MEDIATE EVASION FROM THE NEUTRALIZING ANTIBODY RESPONSE—ARMS RACE BETWEEN VIRUS AND THE HUMORAL IMMUNE RESPONSE

Besides immunological and secondary lymphoid organ topographical factors that might curtail effective T_{FH} responses and thereby induction of neutralizing antibody responses, viral mutation can contribute to the establishment of persistence by escape from imposed immune pressure such as the humoral immune response. RNA viruses are known to evolve upon infection due to a high mutation rate during viral replication with their non-proofreading RNA-dependent RNA polymerase (or reverse transcriptase) and exist as a so-called quasi species in the infected host (143, 144). These high mutation rates allow the rapid adaptation of RNA viruses to changing environments and selective immune pressures (145). Viruses like HIV, HCV and LCMV take advantage of this viral evolution for the establishment of persistence, e.g., by sequential evasion from the adaptive immune response.

In persistent LCMV infection, especially in settings of reduced or absent CD8 T cell responses, escape variants from the neutralizing antibody response emerge that promote persistence of LCMV (15, 16, 146). This escape was mediated by only few amino acid substitutions in the neutralizing epitope contained in GP1 (15). However, LCMV generally has a rather low mutation rate, with 2.6×10^{-4} to 5.5×10^{-5} mutations per round of replication (147), compared with other RNA viruses with 10^{-3} to 10^{-5} misincorporations per copied nucleotide (15, 147, 148). Generally, selection of mutations was reduced or lacking in absence of neutralizing antibodies, indicating positive selection of escape viral variants upon antibody-imposed immune pressure (15). As recently published, escape of LCMV from the neutralizing antibody response also occurs in presence of a normal CD8 T cell response, meaning that neutralization of contemporaneous virus isolates lagged behind neutralization of the inoculating virus (7). This raises the question of how viral diversity is affected in absence of T_{FH} cells. As animals with a conditional depletion of CXCR5^{+/+} T_{FH} cells did not develop effective neutralizing antibodies against

neither the inoculum or contemporaneous virus isolates, circulating antibodies most likely exhibited reduced immune pressure on the neutralizing epitopes (7). One would speculate that viral diversity is more restricted in absence of T_{FH} cells as compared with control situations. Whether this prediction holds will have to be investigated in future studies.

Escape from the neutralizing antibody response and subsequent adaptation of the humoral immune response to new viral variants is more extensively investigated in persistent viral infection with HIV or HCV as compared with persistent LCMV infection. HIV and HCV infection share the common feature that the neutralizing antibody response is at first only directed against the autologous virus, while neutralization of heterologous viral variants by bnab is rather rare and only occurs later during infection (22, 149–152). Moreover, in HIV and HCV infection, the neutralizing antibody response toward the autologous virus usually lags behind the concurrent evolution of the viral quasi species, meaning that antibodies isolated from a given time point generally fail to neutralize contemporaneous virus isolates but are able to neutralize isolates from prior time points (22, 149, 152–154). Thus, during persistent viral infections a molecular arms race is taking place between the virus and the humoral immune response.

Mutations conferring escape are mostly accumulating in variable regions of the viral envelope (env), against which neutralizing antibodies are directed, e.g., the variable loops of HIV gp120 (19, 155) or the hypervariable region (HVR) of HCV (156, 157). Either these variable regions cover more conserved neutralizing epitopes or these regions contain the first neutralizing epitopes as in case of the HVR of HCV (20, 155–157). In addition, shielding of neutralizing epitopes by establishment of a glycan shield *via* mutational introduction of glycosylation sites is used by persistently infecting viruses to hamper binding of neutralizing antibodies by steric hindrance (22, 154, 158–162). Generally, neutralizing antibodies detect deglycosylated forms of the virion better than the glycosylated form as shown for HIV or Arenavirus infections (161, 163–165). Glycans reduce the on-rate of the neutralizing antibody and thereby limit their neutralizing capacity (161). In case of HIV infection, some glycans also increase the flexibility of the variable loops of the envelope protein, thereby increasing the binding entropy for neutralizing antibodies, which is unfavorable (166). Interestingly, however, in some HIV-infected patients, neutralizing antibodies that are able to penetrate the glycan shield by binding one or multiple conserved glycans (e.g., glycans at position N332 or N301) and simultaneously to gp120 protein residues (167–171) were elicited. This clearly shows that the humoral immune response is in principle able to develop antibodies that are able to bypass mechanisms conferring escape from the neutralizing antibody response.

Yet, in persistent HIV or HCV infections such bnab that bind to more conserved epitopes like glycan patches occur rather seldom (149, 150, 172–175). Most bnab are characterized by a high amount of somatic mutations, long CDRH3 regions and preferential usage of specific heavy and light chains (175–180). A high rate of somatic mutations can be observed in neutralizing antibodies against Arenaviruses like LCMV as well (11). Precursors of bnabs can be identified already early during the

virus-specific humoral immune response upon persistent HIV infection (171, 181–186). The slow development of such precursors toward a bnab, together with the high quantity of somatic mutations, indicates that neutralizing antibodies mature over a prolonged period of time in the GC, including selection by T_{FH} cells to develop the necessary neutralization breadth. Factors curtailing the GC response as described earlier might well contribute to the impaired or delayed emergence of such bnab. Furthermore, to allow the continued development of bnab in the GC response, their evolution/selection has to occur against viral variants that do not undergo complete viral escape from these bnab precursors (187).

Interestingly, diversity of the viral variants and the viral load influence the development of bnabs in HIV infection. Prolonged viremia and a higher diversity of the env are positively correlated with their induction (172, 173, 188–192). However, it is still a matter of discussion whether early diversity of the env (189, 190, 192), as, for example, achieved by superinfections (192) or a high diversity of the contemporaneous env genes is correlated with the emergence of bnab (193). Nevertheless, diversification of the viral variants is often observed before the onset of bnab responses (183, 184). Analogous, protracted viremia in persistent infections with Arenaviruses like LCMV is favorable for the induction of the neutralizing antibody response (11).

So far, little is known about the overall evolution of the LCMV-specific antibody response over the course of a chronic infection. Sustained T_{FH} activity is crucial for the eventual emergence of neutralizing antibodies (7). However, how this sustained T_{FH} activity supports the emergence of neutralizing antibodies is unclear. It could either be *via* continuous rounds of SHM and selection of B cells which would eventually give rise to antibodies with neutralizing capacity. Alternatively, T_{FH} cells might be required for continued recruitment of new B cell clones into the GC response, thereby contributing to an overall broadening of the antibody repertoire. Interestingly, a recent vaccination study in humans repetitively exposed to the malaria parasite *Plasmodium falciparum* revealed that selection of potent B cell precursors from the naïve or memory pool contributed more efficiently to a potent antibody response to a complex antigen than the process of affinity maturation (194).

In the context of a chronic viral infection, it would be interesting to elucidate how viral diversity is reflected/presented in the GC response, leading to selection of the precursors of B cells producing neutralizing antibodies. Generally, GC B cells are dependent on taking up antigen from FDCs for affinity selection (76). However, whether this holds true in a setting with abundant free viral antigen during persistent viral infection still has to be determined. Interestingly, however, FDCs have been shown to be archives of viral quasi species upon HIV infection (195, 196), which would indicate constant binding of viral variants and their presentation. This would suggest that FDCs could also present the newest contemporaneous viral variants to B cells, which are then selected according to their affinity toward these variants. Yet, it remains unclear how fast the turnover rate of antigen presented by FDCs is in the setting of a chronic viral infection, which, in case of slow turnover, might lead to delays in the selection of B cells against the newest contemporaneous viral variants.

Moreover, emergence of bnab upon persistent HIV infection is also determined by the rate at which somatic mutations are acquired by B cell clones. For some bnab families it has been determined that the mutation rate was faster than that of the virus (181, 197), which enabled the host to “overtake” the viral evolution and develop an effective neutralizing antibody response. Concerning the role of T_{FH} cells in the selection process of B cells producing such neutralizing antibodies, it has been established recently that the interaction intensity between T_{FH} cells and GC B cells determines the quantity of proliferation rounds and therefore the quantity of somatic mutations a B cell can acquire (198). Therefore, it would be of interest to determine the influence of T_{FH} cells on the mutation rate of such B cell clones. This could be achieved using the novel *in vivo* experimental model that allows conditional depletion of T_{FH} cells upon persistent LCMV infection (7). Virus-specific plasma cells, developed in presence or absence of T_{FH} cells, could be isolated at different time points pi, and the quantity of somatic mutations in the variable regions of heavy and light chains could be determined by NGS. Isolating contemporaneous virus isolates at the same time point and determining the sequences of their neutralizing epitopes by NGS could be used to relate the evolution kinetics of virus-specific B cells to the evolution of the virus. This approach could also be employed to determine whether the observed preferential usage of specific heavy and light chains by neutralizing antibodies is influenced by the absence of T_{FH} cells. In absence of continuous T_{FH} activity, one could conjecture that the overall diversity of B cell clones is increased, as the selection process is most likely much less stringent in absence of T_{FH} cells, and the overall frequency of somatic mutations in B cells might be reduced due to insufficient selection and consecutive rounds of SHM.

DOES ACCUMULATION OF T_{FH} CELLS CONTRIBUTE TO DYSREGULATED B CELL RESPONSES UPON PERSISTENT VIRAL INFECTION?

During persistent viral infections with LCMV, HIV, SIV, or HCV, dysregulated B cell responses are observed. This includes the induction of hypergammaglobulinemia and polyclonal B cell activation, resulting in the emergence of seemingly virus-unspecific antibodies and in some cases even autoimmune reactive antibodies (199–205). However, in a recent study examining antibody responses toward *Salmonella Typhimurium* infection, it was shown that the seemingly predominantly *Salmonella*-unspecific antibody response was in fact of very low affinity toward *Salmonella* that increased due to affinity maturation in extra-follicular patches (206). Therefore, it would be interesting to investigate whether unspecific antibody responses elicited upon persistent viral infections might also display very low (undetectable in commonly used read-outs) affinities for the virus, which might improve upon affinity maturation and then allows recruitment into the virus-specific antibody response.

The described B cell dysfunctions have been further linked to the delayed appearance of neutralizing antibodies and in the context of persistent LCMV infection have been shown

to be dependent on CD4 T cell help to cognate B cells *via* CD40:CD40L signals (13, 202, 207). It is believed that the virus-unspecific B cells acquire viral antigen from the environment and present it *via* their surface MHC II molecules to cognate CD4 T cells. How exactly virus-unspecific B cells acquire viral antigen to present to CD4 T cells and whether they might require signals *via* their BCR to become activated is not fully elucidated. In the setting of persistent LCMV infection, uptake of antigen by LCMV-unspecific B cells is independent of complement receptors (CRs) and FcγR, as knockout mice still display hypergammaglobulinemia (202). A recent study showed in the setting of an acute disseminated encephalomyelitis model with influenza infection, that uptake of the self-antigen myelin oligodendrocyte glycoprotein (MOG) *via* the BCR could occur concurrent with influenza hemagglutinin (HA). This led to the simultaneous presentation of MOG and HA on the MHC II surface molecules of MOG-specific B cells and subsequently their activation *via* HA-specific CD4 T cells (208). This scenario could serve as explanation for the activation of self-reactive B cells in the setting of persistent viral infections and would also indicate participation of BCR signaling pathways. However, this model does not account for virus-unspecific antibody responses toward non-self-antigens, such as for instance against the hapten nitrophenol (202). Another possible pathway that has been proposed to contribute to the uptake of viral antigen by virus-unspecific B cells in the setting of persistent viral infections is pinocytosis (202). Assumingly, due to the high viral burden, the concentration of viral particles and therefore viral antigen would be sufficient to induce sufficient uptake *via* this mechanism from the environment.

Regarding the contribution of T_{FH} cells to dysregulated B cell responses, it has been shown before in settings of autoimmunity that prolonged maintenance of T_{FH} cells, and therefore prolonged maintenance of GC B cells, is one cause for the emergence of autoreactive antibodies (58, 209–213). The selection threshold is lowered in GCs when T_{FH} numbers are increased; thereby permitting the survival of low affinity and self-reactive B cells (214)—a situation that is met during persistent viral infections.

Analogous, in HIV and SIV infection, the expansion of T_{FH} cells observed in LNs of infected individuals correlated with hypergammaglobulinemia and polyclonal B cell activation as well as the deletion of circulating memory B cells (42, 43, 215). Treatment of HIV-infected individuals with antiretroviral therapy reduced T_{FH} cell numbers and at the same time B cell dysfunctions (42, 200), which indicates a connection between expansion of the T_{FH} cell population in persistent HIV and SIV infections and dysregulated B cell responses. Similarly, in persistent HBV infection, the frequency of cT_{FH} cells correlated with the emergence of autoantibodies (205).

ARE THERE ORGAN-SPECIFIC DIFFERENCES IN T_{FH} CELL EXPANSION AND FUNCTION?

It also should be considered when discussing T_{FH} accumulation and its impact on the antibody response that organ-specific

differences might exist in specific persistent viral infections. This has been recently addressed in the context of SIV infection (107). Most studies upon persistent SIV and HIV infection have been conducted in blood or LN samples of infected animals/patients. Yet, recently, T_{FH} responses have been analyzed in spleens of SIV-infected RMs (107). In contrast to results obtained from LNs of SIV-infected RMs, the T_{FH} cell frequency in spleen was drastically reduced already in the acute phase of SIV infection as compared with healthy animals. This phenomenon was maintained in the persistent phase of SIV infection. In addition, T_{FH} cells in spleen of SIV-infected RMs expressed less of the T_{FH}-associated transcription factors Bcl-6 and c-Maf and instead upregulated transcription factors that counter-regulate T_{FH} cell fate, i.e., Krüppel-like factor-2. This decrease in T_{FH} cell frequency was further associated with reduced titers of SIV-specific IgG antibodies (107). However, T_{FH} frequencies were similar or elevated in LNs of these infected RM as compared with healthy animals and in accordance with previous reports (43, 107, 215). Interestingly, the depletion of T_{FH} cells in the spleen of SIV-infected RMs occurred in the context of severe destruction of the splenic architecture (107). Therefore, it might be possible that differences concerning the preservation of the lymphoid tissue could account for the observed organ-specific differences. Probably, due to the severe destruction of splenic architecture, SIV-infected T_{FH} cells might have enhanced contact with cytotoxic CD8 T cells in the acute phase of infection, which might cause deletion of T_{FH} cells in the spleen. Possibly, also differences in the recruitment of effector cells or different cytokine milieu in the LN and the spleen might influence the maintenance of T_{FH} cells upon SIV infection.

Therefore, organ-specific differences in T_{FH} cell frequency and function have to be taken into consideration as together they might account for the outcome of the virus-specific antibody response.

CONCLUDING REMARKS

Follicular T helper cell function and optimal interactions between T_{FH} cells and cognate B cells often are hampered during persistent viral infections due to several factors. These include sustained increase of T_{FH} cells, leading to non-specific B cell activation and hypergammaglobulinemia at the expense of virus-specific antibodies, destruction of the lymphoid tissue architecture, B cell exhaustion, skewed ratios of regulatory cells to T_{FH}/GC B cells or in case of HIV/SIV infection T_{FH} cells being directly infected. Due to these dysregulations, protective virus-specific antibody responses are delayed. Moreover, viruses use different mechanisms to evade recognition by antibodies using, e.g., variable loops or glycan shields to protect neutralizing epitopes. Furthermore, constant viral evolution leads to continued selection of escape variants upon exerted pressure by neutralizing antibodies, which fuels a molecular arms race between virus and the humoral immune response.

Nevertheless, it is clear that sustained activity of T_{FH} cells is essential for the induction of neutralizing, protective antibody

responses upon persistent viral infection and that the eventual emergence of these antibodies can afford control of the persistent infection in absence of overt immunopathology.

Therefore, targeting mechanisms that promote optimal T_{FH} cell function and interactions with cognate B cells as well as understanding the underlying mechanisms of the arms race between virus and humoral immune response might serve to improve the induction of neutralizing antibody responses and reduce B cell dysfunctions, thereby improving control of persistent viral infections.

REFERENCES

- Wherry EJ. T cell exhaustion. *Nat Immunol* (2011) 12(6):492–9. doi:10.1038/nri.2035
- Wherry EJ, Kurachi M. Molecular and cellular insights into T cell exhaustion. *Nat Rev Immunol* (2015) 15(8):486–99. doi:10.1038/nri3862
- Vella LA, Herati RS, Wherry EJ. CD4(+) T cell differentiation in chronic viral infections: the T_{fh} perspective. *Trends Mol Med* (2017) 23(12):1072–87. doi:10.1016/j.molmed.2017.10.001
- Hangartner L, Zellweger RM, Giobbi M, Weber J, Eschli B, McCoy KD, et al. Nonneutralizing antibodies binding to the surface glycoprotein of lymphocytic choriomeningitis virus reduce early virus spread. *J Exp Med* (2006) 203(8):2033–42. doi:10.1084/jem.20051557
- Hangartner L, Zinkernagel RM, Hangartner H. Antiviral antibody responses: the two extremes of a wide spectrum. *Nat Rev Immunol* (2006) 6(3):231–43. doi:10.1038/nri1783
- Berghaler A, Flatz L, Hegazy AN, Johnson S, Horvath E, Löhning M, et al. Viral replicative capacity is the primary determinant of lymphocytic choriomeningitis virus persistence and immunosuppression. *Proc Natl Acad Sci U S A* (2010) 107(50):21641–6. doi:10.1073/pnas.1011998107
- Greczmiel U, Krautler NJ, Pedrioli A, Bartsch I, Agnellini P, Bedenikovic G, et al. Sustained T follicular helper cell response is essential for control of chronic viral infection. *Sci Immunol* (2017) 2(18):eaam8686. doi:10.1126/sciimmunol.aam8686
- Harker JA, Lewis GM, Mack L, Zuniga EI. Late interleukin-6 escalates T follicular helper cell responses and controls a chronic viral infection. *Science* (2011) 334(6057):825–9. doi:10.1126/science.1208421
- Cook KD, Shpargel KB, Starmer J, Whitfield-Larry F, Conley B, Allard DE, et al. T follicular helper cell-dependent clearance of a persistent virus infection requires T cell expression of the histone demethylase UTX. *Immunity* (2015) 43(4):703–14. doi:10.1016/j.immuni.2015.09.002
- Harker JA, Dolgote A, Zuniga EI. Cell-intrinsic IL-27 and gp130 cytokine receptor signaling regulates virus-specific CD4(+) T cell responses and viral control during chronic infection. *Immunity* (2013) 39(3):548–59. doi:10.1016/j.immuni.2013.08.010
- Eschli B, Zellweger RM, Wepf A, Lang KS, Quirin K, Weber J, et al. Early antibodies specific for the neutralizing epitope on the receptor binding subunit of the lymphocytic choriomeningitis virus glycoprotein fail to neutralize the virus. *J Virol* (2007) 81(21):11650–7. doi:10.1128/JVI.00955-07
- Murira A, Lapiere P, Lamarre A. Chapter three – evolution of the humoral response during HCV infection: theories on the origin of broadly neutralizing antibodies and implications for vaccine design. *Adv Immunol* (2016) 129:55–107. doi:10.1016/bs.ai.2015.09.004
- Recher M, Lang KS, Hunziker L, Freigang S, Eschli B, Harris NL, et al. Deliberate removal of T cell help improves virus-neutralizing antibody production. *Nat Immunol* (2004) 5(9):934–42. doi:10.1038/nri1102
- Coutelier JP, Johnston SJ, El Idrissi MEA, Pfau CJ. Involvement of CD4+ cells in lymphocytic choriomeningitis virus-induced autoimmune anaemia and hypergammaglobulinaemia. *J Autoimmun* (1994) 7(5):589–99. doi:10.1006/jaut.1994.1043
- Ciurea A, Hunziker L, Zinkernagel RM, Hangartner H. Viral escape from the neutralizing antibody response: the lymphocytic choriomeningitis virus model. *Immunogenetics* (2001) 53(3):185–9. doi:10.1007/s002510100314

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

FUNDING

This work was supported by ETH Zürich, the Swiss National Science Foundation (grant no. 310030_146140 and 310030_166078 to AO), and the Promedica Foundation.

- Ciurea A, Klenerman P, Hunziker L, Horvath E, Senn BM, Ochsenbein AF, et al. Viral persistence in vivo through selection of neutralizing antibody-escape variants. *Proc Natl Acad Sci U S A* (2000) 97(6):2749–54. doi:10.1073/pnas.040558797
- Pircher H, Moskopidis D, Rohrer U, Bürki K, Hengartner H, Zinkernagel RM. Viral escape by selection of cytotoxic T cell-resistant virus variants in vivo. *Nature* (1990) 346:629. doi:10.1038/346629a0
- Geiss YD. Ursula: catch me if you can – the race between HIV and neutralizing antibodies. *AIDS Rev* (2015) 17(2):107–13.
- Burton DR, Mascola JR. Antibody responses to envelope glycoproteins in HIV-1 infection. *Nat Immunol* (2015) 16(6):571–6. doi:10.1038/nri.3158
- Burton DR, Stanfield RL, Wilson IA. Antibody vs. HIV in a clash of evolutionary titans. *Proc Natl Acad Sci U S A* (2005) 102(42):14943–8. doi:10.1073/pnas.0505126102
- Bukh J. The history of hepatitis C virus (HCV): basic research reveals unique features in phylogeny, evolution and the viral life cycle with new perspectives for epidemic control. *J Hepatol* (2016) 65(1 Suppl):S2–21. doi:10.1016/j.jhep.2016.07.035
- Richman DD, Wrinn T, Little SJ, Petropoulos CJ. Rapid evolution of the neutralizing antibody response to HIV type 1 infection. *Proc Natl Acad Sci U S A* (2003) 100(7):4144–9. doi:10.1073/pnas.0630530100
- Vinuesa CG, Linterman MA, Yu D, MacLennan ICM. Follicular helper T cells. *Annu Rev Immunol* (2016) 34(1):335–68. doi:10.1146/annurev-immunol-041015-055605
- Johnston RJ, Poholek AC, DiToro D, Yusuf I, Eto D, Barnett B, et al. Bcl6 and Blimp-1 are reciprocal and antagonistic regulators of T follicular helper cell differentiation. *Science* (2009) 325(5943):1006–10. doi:10.1126/science.1175870
- Nurieva RI, Chung Y, Martinez GJ, Yang XO, Tanaka S, Matskevitch TD, et al. Bcl6 mediates the development of T follicular helper cells. *Science* (2009) 325(5943):1001–5. doi:10.1126/science.1176676
- Yu D, Rao S, Tsai LM, Lee SK, He Y, Sutcliffe EL, et al. The transcriptional repressor BCL-6 directs T follicular helper cell lineage commitment. *Immunity* (2009) 31(3):457–68. doi:10.1016/j.immuni.2009.07.002
- Ansel KM, McHeyzer-Williams LJ, Ngo VN, McHeyzer-Williams MG, Cyster JG. In vivo-activated Cd4 T cells upregulate Cxcr chemokine receptor 5 and reprogram their response to lymphoid chemokines. *J Exp Med* (1999) 190(8):1123–34. doi:10.1084/jem.190.8.1123
- Breitfeld D, Ohl L, Kremmer E, Ellwart J, Sallusto F, Lipp M, et al. Follicular B helper T cells express Cxcr chemokine receptor 5, localize to B cell follicles, and support immunoglobulin production. *J Exp Med* (2000) 192(11):1545–52. doi:10.1084/jem.192.11.1545
- Schaerli P, Willmann K, Lang AB, Lipp M, Loetscher P, Moser B. Cxcr chemokine receptor 5 expression defines follicular homing T cells with B cell helper function. *J Exp Med* (2000) 192(11):1553–62. doi:10.1084/jem.192.11.1553
- Lee SK, Rigby RJ, Zotos D, Tsai LM, Kawamoto S, Marshall JL, et al. B cell priming for extrafollicular antibody responses requires Bcl-6 expression by T cells. *J Exp Med* (2011) 208(7):1377–88. doi:10.1084/jem.20102065
- MacLennan ICM, Toellner K-M, Cunningham AF, Serre K, Sze DMY, Zúñiga E, et al. Extrafollicular antibody responses. *Immunol Rev* (2003) 194(1):8–18. doi:10.1034/j.1600-065X.2003.00058.x
- O'Connor BP, Vogel LA, Zhang W, Loo W, Shneider D, Lind EF, et al. Imprinting the fate of antigen-reactive B cells through the affinity of the B cell receptor. *J Immunol* (2006) 177(11):7723–32. doi:10.4049/jimmunol.177.11.7723

33. Taylor JJ, Pape KA, Jenkins MK. A germinal center-independent pathway generates unswitched memory B cells early in the primary response. *J Exp Med* (2012) 209(3):597–606. doi:10.1084/jem.20111696
34. Barnett LG, Simkins HMA, Barnett BE, Korn LL, Johnson AL, Wherry EJ, et al. B cell antigen presentation in the initiation of follicular helper T cell and germinal center differentiation. *J Immunol* (2014) 192(8):3607–17. doi:10.4049/jimmunol.1301284
35. Deenick EK, Chan A, Ma CS, Gatto D, Schwartzberg PL, Brink R, et al. Follicular helper T cell differentiation requires continuous antigen presentation that is independent of unique B cell signaling. *Immunity* (2010) 33(2):241–53. doi:10.1016/j.immuni.2010.07.015
36. Goenka R, Barnett LG, Silver JS, O'Neill PJ, Hunter CA, Cancro MP, et al. Cutting edge: dendritic cell-restricted antigen presentation initiates the follicular helper T cell program but cannot complete ultimate effector differentiation. *J Immunol* (2011) 187(3):1091–5. doi:10.4049/jimmunol.1100853
37. Kitano M, Moriyama S, Ando Y, Hikida M, Mori Y, Kurosaki T, et al. Bcl6 protein expression shapes pre-germinal center B cell dynamics and follicular helper T cell heterogeneity. *Immunity* (2011) 34(6):961–72. doi:10.1016/j.immuni.2011.03.025
38. Fahey LM, Wilson EB, Elsaesser H, Fistonich CD, McGavern DB, Brooks DG. Viral persistence redirects CD4 T cell differentiation toward T follicular helper cells. *J Exp Med* (2011) 208(5):987–99. doi:10.1084/jem.20101773
39. Shulman Z, Gitlin AD, Weinstein JS, Lainez B, Esplugues E, Flavell RA, et al. Dynamic signaling by T follicular helper cells during germinal center B cell selection. *Science* (2014) 345(6200):1058–62. doi:10.1126/science.1257861
40. Liu D, Xu H, Shih C, Wan Z, Ma X, Ma W, et al. T-B-cell entanglement and ICOSL-driven feed-forward regulation of germinal centre reaction. *Nature* (2015) 517(7533):214–8. doi:10.1038/nature13803
41. Vitoria GD, Nussenzweig MC. Germinal centers. *Annu Rev Immunol* (2012) 30(1):429–57. doi:10.1146/annurev-immunol-020711-075032
42. Lindqvist M, van Lunzen J, Soghoian DZ, Kuhl BD, Ransinghe S, Kranias G, et al. Expansion of HIV-specific T follicular helper cells in chronic HIV infection. *J Clin Invest* (2012) 122(9):3271–80. doi:10.1172/JCI64314
43. Petrovas C, Yamamoto T, Gerner MY, Boswell KL, Wloka K, Smith EC, et al. CD4 T follicular helper cell dynamics during SIV infection. *J Clin Invest* (2012) 122(9):3281–94. doi:10.1172/JCI63039
44. Osokine I, Snell LM, Cunningham CR, Yamada DH, Wilson EB, Elsaesser HJ, et al. Type I interferon suppresses de novo virus-specific CD4 Th1 immunity during an established persistent viral infection. *Proc Natl Acad Sci U S A* (2014) 111(20):7409–14. doi:10.1073/pnas.1401662111
45. Kuller LH, Tracy R, Belloso W, Wit SD, Drummond F, Lane HC, et al. Inflammatory and coagulation biomarkers and mortality in patients with HIV infection. *PLoS Med* (2008) 5(10):e203. doi:10.1371/journal.pmed.0050203
46. Baumjohann D, Preite S, Reboldi A, Ronchi F, Ansel KM, Lanzavecchia A, et al. Persistent antigen and germinal center B cells sustain T follicular helper cell responses and phenotype. *Immunity* (2013) 38(3):596–605. doi:10.1016/j.immuni.2012.11.020
47. Butler NS, Kulu DI. The regulation of T follicular helper responses during infection. *Curr Opin Immunol* (2015) 34:68–74. doi:10.1016/j.coi.2015.02.007
48. Chakarov S, Fazilleau N. Monocyte-derived dendritic cells promote T follicular helper cell differentiation. *EMBO Mol Med* (2014) 6(5):590–603. doi:10.1002/emmm.201403841
49. Eto D, Lao C, DiToro D, Barnett B, Escobar TC, Kageyama R, et al. IL-21 and IL-6 are critical for different aspects of B cell immunity and redundantly induce optimal follicular helper CD4 T cell (T_{fh}) differentiation. *PLoS One* (2011) 6(3):e17739. doi:10.1371/journal.pone.0017739
50. Karnowski A, Chevrier S, Belz GT, Mount A, Emslie D, D'Costa K, et al. B and T cells collaborate in antiviral responses via IL-6, IL-21, and transcriptional activator and coactivator, Oct2 and OBF-1. *J Exp Med* (2012) 209(11):2049–64. doi:10.1084/jem.20111504
51. Hatzi K, Nance JP, Kroenke MA, Bothwell M, Haddad EK, Melnick A, et al. BCL6 orchestrates T_{fh} cell differentiation via multiple distinct mechanisms. *J Exp Med* (2015) 212(4):539–53. doi:10.1084/jem.20141380
52. Weber JP, Fuhrmann F, Feist RK, Lahmann A, Al Baz MS, Gentz L-J, et al. ICOS maintains the T follicular helper cell phenotype by down-regulating Krüppel-like factor 2. *J Exp Med* (2015) 212(2):217–33. doi:10.1084/jem.20141432
53. Lee JY, Skon CN, Lee YJ, Oh S, Taylor JJ, Malhotra D, et al. The transcription factor KLF2 restrains CD4+ T follicular helper cell differentiation. *Immunity* (2015) 42(2):252–64. doi:10.1016/j.immuni.2015.01.013
54. Liu X, Chen X, Zhong B, Wang A, Wang X, Chu F, et al. Transcription factor achaete-scute homologue 2 initiates follicular T-helper-cell development. *Nature* (2014) 507(7493):513–8. doi:10.1038/nature12910
55. Chtanova T, Tangye SG, Newton R, Frank N, Hodge MR, Rolph MS, et al. T follicular helper cells express a distinctive transcriptional profile, reflecting their role as non-Th1/Th2 effector cells that provide help for B cells. *J Immunol* (2004) 173(1):68–78. doi:10.4049/jimmunol.173.1.68
56. Haynes NM, Allen CDC, Lesley R, Ansel KM, Killeen N, Cyster JG. Role of CXCR5 and CCR7 in follicular Th cell positioning and appearance of a programmed cell death gene-1-high germinal center-associated subpopulation. *J Immunol* (2007) 179(8):5099–108. doi:10.4049/jimmunol.179.8.5099
57. Reinhardt RL, Liang H-E, Locksley RM. Cytokine-secreting follicular T cells shape the antibody repertoire. *Nat Immunol* (2009) 10(4):385–93. doi:10.1038/ni.1715
58. Choi YS, Kageyama R, Eto D, Escobar TC, Johnston RJ, Monticelli L, et al. ICOS receptor instructs T follicular helper cell versus effector cell differentiation via induction of the transcriptional repressor Bcl6. *Immunity* (2011) 34(6):932–46. doi:10.1016/j.immuni.2011.03.023
59. Kim CH, Rott LS, Clark-Lewis I, Campbell DJ, Wu L, Butcher EC. Subspecialization of Cxcr5+ T cells: B helper activity is focused in a germinal center-localized subset of Cxcr5+ T cells. *J Exp Med* (2001) 193(12):1373–82. doi:10.1084/jem.193.12.1373
60. Suan D, Nguyen A, Moran I, Bourne K, Hermes JR, Arshi M, et al. T follicular helper cells have distinct modes of migration and molecular signatures in naive and memory immune responses. *Immunity* (2015) 42(4):704–18. doi:10.1016/j.immuni.2015.03.002
61. Luthje K, Kallies A, Shimohakamada Y, Belz GT, Light A, Tarlinton DM, et al. The development and fate of follicular helper T cells defined by an IL-21 reporter mouse. *Nat Immunol* (2012) 13(5):491–8. doi:10.1038/ni.2261
62. Weber JP, Fuhrmann F, Hutloff A. T-follicular helper cells survive as long-term memory cells. *Eur J Immunol* (2012) 42(8):1981–8. doi:10.1002/eji.201242540
63. Chung Y, Tanaka S, Chu F, Nurieva RI, Martinez GJ, Rawal S, et al. Follicular regulatory T cells expressing Foxp3 and Bcl-6 suppress germinal center reactions. *Nat Med* (2011) 17(8):983–8. doi:10.1038/nm.2426
64. Linterman MA, Pierson W, Lee SK, Kallies A, Kawamoto S, Rayner TF, et al. Foxp3+ follicular regulatory T cells control the germinal center response. *Nat Med* (2011) 17(8):975–82. doi:10.1038/nm.2425
65. Wollenberg I, Agua-Doce A, Hernández A, Almeida C, Oliveira VG, Faro J, et al. Regulation of the germinal center reaction by Foxp3+ follicular regulatory T cells. *J Immunol* (2011) 187(9):4553–60. doi:10.4049/jimmunol.1101328
66. Coffey F, Alabyev B, Manser T. Initial clonal expansion of germinal center B cells takes place at the perimeter of follicles. *Immunity* (2009) 30(4):599–609. doi:10.1016/j.immuni.2009.01.011
67. Kerfoot SM, Yaari G, Patel JR, Johnson KL, Gonzalez DG, Kleinstein SH, et al. Germinal center B cell and T follicular helper cell development initiates in the interfollicular zone. *Immunity* (2011) 34(6):947–60. doi:10.1016/j.immuni.2011.03.024
68. Okada T, Miller MJ, Parker I, Krummel MF, Neighbors M, Hartley SB, et al. Antigen-engaged B cells undergo chemotaxis toward the T zone and form motile conjugates with helper T cells. *PLoS Biol* (2005) 3(6):e150. doi:10.1371/journal.pbio.0030150
69. Paus D, Phan TG, Chan TD, Gardam S, Basten A, Brink R. Antigen recognition strength regulates the choice between extrafollicular plasma cell and germinal center B cell differentiation. *J Exp Med* (2006) 203(4):1081–91. doi:10.1084/jem.20060087
70. Green JA, Suzuki K, Cho B, Willison LD, Palmer D, Allen CDC, et al. The sphingosine 1-phosphate receptor SIP2 maintains the homeostasis of germinal center B cells and promotes niche confinement. *Nat Immunol* (2011) 12(7):672–80. doi:10.1038/ni.2047
71. Okada T, Cyster JG. B cell migration and interactions in the early phase of antibody responses. *Curr Opin Immunol* (2006) 18(3):278–85. doi:10.1016/j.coi.2006.02.005

72. Allen CDC, Ansel KM, Low C, Lesley R, Tamamura H, Fujii N, et al. Germinal center dark and light zone organization is mediated by CXCR4 and CXCR5. *Nat Immunol* (2004) 5(9):943–52. doi:10.1038/ni1100
73. Allen CDC, Okada T, Cyster JG. Germinal-center organization and cellular dynamics. *Immunity* (2007) 27(2):190–202. doi:10.1016/j.immuni.2007.07.009
74. Allen CDC, Okada T, Tang HL, Cyster JG. Imaging of germinal center selection events during affinity maturation. *Science* (2007) 315(5811):528–31. doi:10.1126/science.1136736
75. Vitorica GD, Schwickert TA, Fooksman DR, Kamphorst AO, Meyer-Hermann M, Dustin ML, et al. Germinal center dynamics revealed by multiphoton microscopy with a photoactivatable fluorescent reporter. *Cell* (2010) 143(4):592–605. doi:10.1016/j.cell.2010.10.032
76. Mesin L, Ersching J, Vitorica GD. Germinal center B cell dynamics. *Immunity* (2016) 45(3):471–82. doi:10.1016/j.immuni.2016.09.001
77. Muramatsu M, Kinoshita K, Fagarasan S, Yamada S, Shinkai Y, Honjo T. Class switch recombination and hypermutation require activation-induced cytidine deaminase (AID), a potential RNA editing enzyme. *Cell* (2000) 102(5):553–63. doi:10.1016/S0092-8674(00)00078-7
78. Muramatsu M, Sankaranand VS, Anant S, Sugai M, Kinoshita K, Davidson NO, et al. Specific expression of activation-induced cytidine deaminase (AID), a novel member of the RNA-editing deaminase family in germinal center B cells. *J Biol Chem* (1999) 274(26):18470–6. doi:10.1074/jbc.274.26.18470
79. Teng G, Papavasiliou FN. Immunoglobulin somatic hypermutation. *Annu Rev Genet* (2007) 41:107–20. doi:10.1146/annurev.genet.41.110306.130340
80. Batista FD, Neuberger MS. B cells extract and present immobilized antigen: implications for affinity discrimination. *EMBO J* (2000) 19(4):513–20. doi:10.1093/emboj/19.4.513
81. Yoshino T, Kondo E, Cao L, Takahashi K, Hayashi K, Nomura S, et al. Inverse expression of bcl-2 protein and Fas antigen in lymphoblasts in peripheral lymph nodes and activated peripheral blood T and B lymphocytes. *Blood* (1994) 83(7):1856–61.
82. Shinnakasu R, Inoue T, Kometani K, Moriyama S, Adachi Y, Nakayama M, et al. Regulated selection of germinal-center cells into the memory B cell compartment. *Nat Immunol* (2016) 17(7):861–9. doi:10.1038/ni.3460
83. Phan TG, Paus D, Chan TD, Turner ML, Nutt SL, Basten A, et al. High affinity germinal center B cells are actively selected into the plasma cell compartment. *J Exp Med* (2006) 203(11):2419–24. doi:10.1084/jem.20061254
84. Smith KGC, Light A, Nossal GJV, Tarlinton DM. The extent of affinity maturation differs between the memory and antibody-forming cell compartments in the primary immune response. *EMBO J* (1997) 16(11):2996–3006. doi:10.1093/emboj/16.11.2996
85. Seiler P, Kalinke U, Rüllicke T, Bucher EM, Böse C, Zinkernagel RM, et al. Enhanced virus clearance by early inducible lymphocytic choriomeningitis virus-neutralizing antibodies in immunoglobulin-transgenic mice. *J Virol* (1998) 72(3):2253–8.
86. McIlwain DR, Grusdat M, Pozdeev VI, Xu HC, Shinde P, Reardon C, et al. T-cell STAT3 is required for the maintenance of humoral immunity to LCMV. *Eur J Immunol* (2015) 45(2):418–27. doi:10.1002/eji.201445060
87. Clouthier DL, Zhou AC, Wortzman ME, Luft O, Levy GA, Watts TH. GITR intrinsically sustains early type 1 and late follicular helper CD4 T cell accumulation to control a chronic viral infection. *PLoS Pathog* (2015) 11(1):e1004517. doi:10.1371/journal.ppat.1004517
88. Kang SG, Liu WH, Lu P, Jin HY, Lim HW, Shepherd J, et al. MicroRNAs of the miR-17 approximately 92 family are critical regulators of T(FH) differentiation. *Nat Immunol* (2013) 14(8):849–57. doi:10.1038/ni.2648
89. Cook KD, Kline HC, Whitmire JK. NK cells inhibit humoral immunity by reducing the abundance of CD4+ T follicular helper cells during a chronic virus infection. *J Leukoc Biol* (2015) 98(2):153–62. doi:10.1189/jlb.4HI1214-594R
90. Velu V, Mylvaganam GH, Gangadhara S, Hong JJ, Iyer SS, Gumber S, et al. Induction of Th1-biased T follicular helper (Tfh) cells in lymphoid tissues during chronic simian immunodeficiency virus infection defines functionally distinct germinal center Tfh cells. *J Immunol* (2016) 197(5):1832–42. doi:10.4049/jimmunol.1600143
91. Yamamoto T, Lynch RM, Gautam R, Matus-Nicodemus R, Schmidt SD, Boswell KL, et al. Quality and quantity of TFH cells are critical for broad antibody development in SHIVAD8 infection. *Sci Transl Med* (2015) 7(298):298ra120. doi:10.1126/scitranslmed.aab3964
92. Locci M, Havenar-Daughton C, Landais E, Wu J, Kroenke MA, Arlehamn CL, et al. Human circulating PD-1+CXCR3–CXCR5+ memory Tfh cells are highly functional and correlate with broadly neutralizing HIV antibody responses. *Immunity* (2013) 39(4):758–69. doi:10.1016/j.immuni.2013.08.031
93. Buranapraditkun S, Pissani F, Teigler JE, Schultz BT, Alter G, Marovich M, et al. Preservation of peripheral T follicular helper cell function in HIV controllers. *J Virol* (2017) 91(14):e497–417. doi:10.1128/JVI.00497-17
94. Feng J, Lu L, Hua C, Qin L, Zhao P, Wang J, et al. High frequency of CD4+CXCR5+ TFH cells in patients with immune-active chronic hepatitis B. *PLoS One* (2011) 6(7):e21698. doi:10.1371/journal.pone.0021698
95. Cubas R, van Grevenynghe J, Wills S, Kardava L, Santich BH, Buckner CM, et al. Reversible reprogramming of circulating memory T follicular helper cell function during chronic HIV infection. *J Immunol* (2015) 195(12):5625–36. doi:10.4049/jimmunol.1501524
96. Blackburn MJ, Zhong-Min M, Caccuri F, McKinnon K, Schifanello L, Guan Y, et al. Regulatory and helper follicular T cells and antibody avidity to simian immunodeficiency virus glycoprotein 120. *J Immunol* (2015) 195(7):3227–36. doi:10.4049/jimmunol.1402699
97. Hu TT, Song XF, Lei Y, Hu HD, Ren H, Hu P. Expansion of circulating TFH cells and their associated molecules: involvement in the immune landscape in patients with chronic HBV infection. *Virol J* (2014) 11:54. doi:10.1186/1743-422X-11-54
98. Li Y, Ma S, Tang L, Li Y, Wang W, Huang X, et al. Circulating chemokine (C-X-C motif) receptor 5(+) CD4(+) T cells benefit hepatitis B e antigen seroconversion through IL-21 in patients with chronic hepatitis B virus infection. *Hepatology* (2013) 58(4):1277–86. doi:10.1002/hep.26489
99. Boswell KL, Paris R, Boritz E, Ambrozak D, Yamamoto T, Darko S, et al. Loss of circulating CD4 T cells with B cell helper function during chronic HIV infection. *PLoS Pathog* (2014) 10(1):e1003853. doi:10.1371/journal.ppat.1003853
100. Xu H, Wang X, Malam N, Lackner AA, Veazey RS. Persistent simian immunodeficiency virus infection causes ultimate depletion of follicular Th cells in AIDS. *J Immunol* (2015) 195(9):4351–7. doi:10.4049/jimmunol.1501273
101. Haynes BF, Shaw GM, Korber B, Kelsoe G, Sodroski J, Hahn BH, et al. HIV-host interactions: implications for vaccine design. *Cell Host Microbe* (2016) 19(3):292–303. doi:10.1016/j.chom.2016.02.002
102. Klein F, Mouquet H, Dosenovic P, Scheid JF, Scharf L, Nussenzweig MC. Antibodies in HIV-1 vaccine development and therapy. *Science* (2013) 341(6151):1199–204. doi:10.1126/science.1241144
103. Scandella E, Bolinger B, Lattmann E, Miller S, Favre S, Littman DR, et al. Restoration of lymphoid organ integrity through the interaction of lymphoid tissue-inducer cells with stroma of the T cell zone. *Nat Immunol* (2008) 9(6):667–75. doi:10.1038/ni.1605
104. Odermatt B, Eppler M, Leist TP, Hengartner H, Zinkernagel RM. Virus-triggered acquired immunodeficiency by cytotoxic T-cell-dependent destruction of antigen-presenting cells and lymph follicle structure. *Proc Natl Acad Sci U S A* (1991) 88(18):8252–6. doi:10.1073/pnas.88.18.8252
105. Battagay M, Moskopidhis D, Waldner H, Bründler MA, Fung-Leung WP, Mak TW, et al. Impairment and delay of neutralizing antiviral antibody responses by virus-specific cytotoxic T cells. *J Immunol* (1993) 151(10):5408–15.
106. Levesque MC, Moody MA, Hwang K-K, Marshall DJ, Whitesides JF, Amos JD, et al. Polyclonal B cell differentiation and loss of gastrointestinal tract germinal centers in the earliest stages of HIV-1 infection. *PLoS Med* (2009) 6(7):e1000107. doi:10.1371/journal.pmed.1000107
107. Moukambi F, Rabazanahary H, Rodrigues V, Racine G, Robitaille L, Krust B, et al. Early loss of splenic Tfh cells in SIV-infected rhesus macaques. *PLoS Pathog* (2015) 11(12):e1005287. doi:10.1371/journal.ppat.1005287
108. Wang L, Qiu J, Yu L, Hu X, Zhao P, Jiang Y. Increased numbers of CD5+CD19+CD1dhighIL-10+ Bregs, CD4+Foxp3+ Tregs, CD4+CXCR5+Foxp3+ follicular regulatory T (TFR) cells in CHB or CHC patients. *J Transl Med* (2014) 12(1):251. doi:10.1186/s12967-014-0251-9
109. Estes JD, Wietgreffe S, Schacker T, Southern P, Beilman G, Reilly C, et al. Simian immunodeficiency virus-induced lymphatic tissue fibrosis is mediated by transforming growth factor beta 1-positive regulatory T cells and begins in early infection. *J Infect Dis* (2007) 195(4):551–61. doi:10.1086/510852
110. Zeng M, Smith AJ, Wietgreffe SW, Southern PJ, Schacker TW, Reilly CS, et al. Cumulative mechanisms of lymphoid tissue fibrosis and T cell depletion in

- HIV-1 and SIV infections. *J Clin Invest* (2011) 121(3):998–1008. doi:10.1172/JCI45157
111. Wilson EB, Yamada DH, Elsaesser H, Herskovitz J, Deng J, Cheng G, et al. Blockade of chronic type I interferon signaling to control persistent LCMV infection. *Science* (2013) 340(6129):202–7. doi:10.1126/science.1235208
 112. Teijaro JR, Ng C, Lee AM, Sullivan BM, Sheehan KCF, Welch M, et al. Persistent LCMV infection is controlled by blockade of type I interferon signaling. *Science* (2013) 340(6129):207–11. doi:10.1126/science.1235214
 113. Matter MS, Hilmenyuk T, Claus C, Marone R, Schürch C, Tinguely M, et al. Destruction of lymphoid organ architecture and hepatitis caused by CD4+ T cells. *PLoS One* (2011) 6(9):e24772. doi:10.1371/journal.pone.0024772
 114. Song H, Cerny J. Functional heterogeneity of marginal zone B cells revealed by their ability to generate both early antibody-forming cells and germinal centers with hypermutation and memory in response to a T-dependent antigen. *J Exp Med* (2003) 198(12):1923–35. doi:10.1084/jem.20031498
 115. Cinamon G, Matloubian M, Lesneski MJ, Xu Y, Low C, Lu T, et al. Sphingosine 1-phosphate receptor 1 promotes B cell localization in the splenic marginal zone. *Nat Immunol* (2004) 5(7):713–20. doi:10.1038/ni1083
 116. Junt T, Moseman EA, Iannacone M, Massberg S, Lang PA, Boes M, et al. Subcapsular sinus macrophages in lymph nodes clear lymph-borne viruses and present them to antiviral B cells. *Nature* (2007) 450(7166):110–4. doi:10.1038/nature06287
 117. Wilkins BS, Davis Z, Lucas SB, Delsol G, Jones DB. Splenic marginal zone atrophy and progressive CD8+ T-cell lymphocytosis in HIV infection: a study of adult post-mortem spleens from Côte d'Ivoire. *Histopathology* (2003) 42(2):173–85. doi:10.1046/j.1365-2559.2003.01569.x
 118. Kaplan DE, Sugimoto K, Newton K, Valiga ME, Ikeda F, Aytaman A, et al. Discordant role of CD4 T-cell response relative to neutralizing antibody and CD8 T-cell responses in acute hepatitis C. *Gastroenterology* (2007) 132(2):654–66. doi:10.1053/j.gastro.2006.11.044
 119. Frebel H, Richter K, Oxenius A. How chronic viral infections impact on antigen-specific T-cell responses. *Eur J Immunol* (2010) 40(3):654–63. doi:10.1002/eji.200940102
 120. Oxenius A, Zinkernagel RM, Hengartner H. Comparison of activation versus induction of unresponsiveness of virus-specific CD4+ and CD8+ T cells upon acute versus persistent viral infection. *Immunity* (1998) 9(4):449–57. doi:10.1016/S1074-7613(00)80628-7
 121. Fuller MJ, Zajac AJ. Ablation of CD8 and CD4 T cell responses by high viral loads. *J Immunol* (2003) 170(1):477–86. doi:10.4049/jimmunol.170.1.477
 122. Moir S, Fauci AS. B-cell exhaustion in HIV infection: the role of immune activation. *Curr Opin HIV AIDS* (2014) 9(5):472–7. doi:10.1097/COH.0000000000000092
 123. Cubas RA, Mudd JC, Savoye A-L, Perreau M, van Grevenynghe J, Metcalf T, et al. Inadequate T follicular cell help impairs B cell immunity during HIV infection. *Nat Med* (2013) 19(4):494–9. doi:10.1038/nm.3109
 124. Spaan M, Kreeft K, de Graaf GN, Brouwer WP, de Knecht RJ, ten Kate FJW, et al. CD4+CXCR5+ T cells in chronic HCV infection produce less IL-21, yet are efficient at supporting B cell responses. *J Hepatol* (2015) 62(2):303–10. doi:10.1016/j.jhep.2014.09.024
 125. Havenar-Daughton C, Carnathan DG, Torrents de la Peña A, Pauthner M, Briney B, Reiss SM, et al. Direct probing of germinal center responses reveals immunological features and bottlenecks for neutralizing antibody responses to HIV Env trimer. *Cell Rep* (2016) 17(9):2195–209. doi:10.1016/j.celrep.2016.10.085
 126. Chowdhury A, Del Rio PME, Sharp GK, Tribble RP, Amara RR, Chahroudi A, et al. Decreased T follicular regulatory cell/T follicular helper cell (TFH) in simian immunodeficiency virus-infected rhesus macaques may contribute to accumulation of TFH in chronic infection. *J Immunol* (2015) 195(7):3237–47. doi:10.4049/jimmunol.1502269
 127. Miles B, Miller SM, Folkvord JM, Kimball A, Chamanian M, Meditz AL, et al. Follicular regulatory T cells impair follicular T helper cells in HIV and SIV infection. *Nat Commun* (2015) 6:8608. doi:10.1038/ncomms9608
 128. Miles B, Miller SM, Connick E. CD4 T follicular helper and regulatory cell dynamics and function in HIV infection. *Front Immunol* (2016) 7:659. doi:10.3389/fimmu.2016.00659
 129. Preite S, Baumjohann D, Foglierini M, Basso C, Ronchi F, Rodriguez BME, et al. Somatic mutations and affinity maturation are impaired by excessive numbers of T follicular helper cells and restored by Treg cells or memory T cells. *Eur J Immunol* (2015) 45(11):3010–21. doi:10.1002/eji.201545920
 130. Feng C, Cao L-J, Song H-F, Xu P, Chen H, Xu J-C, et al. Expression of PD-L1 on CD4+CD25+Foxp3+ regulatory T cells of patients with chronic HBV infection and its correlation with clinical parameters. *Viral Immunol* (2015) 28(8):418–24. doi:10.1089/vim.2015.0062
 131. Gong Y, Zhao C, Zhao P, Wang M, Zhou G, Han F, et al. Role of IL-10-producing regulatory B cells in chronic hepatitis B virus infection. *Dig Dis Sci* (2015) 60(5):1308–14. doi:10.1007/s10620-014-3358-1
 132. Liu Y, Cheng LS, Wu SD, Wang SQ, Li L, She WM, et al. IL-10-producing regulatory B-cells suppressed effector T-cells but enhanced regulatory T-cells in chronic HBV infection. *Clin Sci* (2016) 130(11):907–19. doi:10.1042/CS20160069
 133. Oxenius A, Bachmann MF, Zinkernagel RM, Hengartner H. Virus-specific major MHC class II-restricted TCR-transgenic mice: effects on humoral and cellular immune responses after viral infection. *Eur J Immunol* (1998) 28(1):390–400. doi:10.1002/(SICI)1521-4141(199801)28:01<390::AID-IMMU390>3.0.CO;2-O
 134. Yin L, Calvo-Calle JM, Cruz J, Newman FK, Frey SE, Ennis FA, et al. CD4+ T cells provide intermolecular help to generate robust antibody responses in vaccinia virus-vaccinated humans. *J Immunol* (2013) 190(12):6023–33. doi:10.4049/jimmunol.1202523
 135. Kohler SL, Pham MN, Folkvord JM, Arends T, Miller SM, Miles B, et al. Germinal center T follicular helper cells are highly permissive to HIV-1 and alter their phenotype during virus replication. *J Immunol* (2016) 196(6):2711–22. doi:10.4049/jimmunol.1502174
 136. Perreau M, Savoye A-L, De Crignis E, Corpataux J-M, Cubas R, Haddad EK, et al. Follicular helper T cells serve as the major CD4 T cell compartment for HIV-1 infection, replication, and production. *J Exp Med* (2013) 210(1):143–56. doi:10.1084/jem.20121932
 137. Xu H, Wang X, Malam N, Aye PP, Alvarez X, Lackner AA, et al. Persistent simian immunodeficiency virus infection drives differentiation, aberrant accumulation, and latent infection of germinal center follicular T helper cells. *J Virol* (2015) 90(3):1578–87. doi:10.1128/JVI.02471-15
 138. Miller SM, Miles B, Guo K, Folkvord J, Meditz AL, McCarter MD, et al. Follicular regulatory T cells are highly permissive to r5-tropic HIV-1. *J Virol* (2017) 91(17):e430–417. doi:10.1128/JVI.00430-17
 139. Fukazawa Y, Lum R, Okoye AA, Park H, Matsuda K, Bae JY, et al. B cell follicle sanctuary permits persistent productive simian immunodeficiency virus infection in elite controllers. *Nat Med* (2015) 21(2):132–9. doi:10.1038/nm.3781
 140. Connick E, Mattila T, Folkvord JM, Schlichtemeier R, Meditz AL, Ray MG, et al. CTL fail to accumulate at sites of HIV-1 replication in lymphoid tissue. *J Immunol* (2007) 178(11):6975–83. doi:10.4049/jimmunol.178.11.6975
 141. Mylvaganam GH, Rios D, Abdelaal HM, Iyer S, Tharp G, Mavinger M, et al. Dynamics of SIV-specific CXCR5+ CD8 T cells during chronic SIV infection. *Proc Natl Acad Sci U S A* (2017) 114(8):1976–81. doi:10.1073/pnas.1621481114
 142. Miles B, Miller SM, Folkvord JM, Levy DN, Rakasz EG, Skinner PJ, et al. Follicular regulatory CD8 T cells impair the germinal center response in SIV and ex vivo HIV infection. *PLoS Pathog* (2016) 12(10):e1005924. doi:10.1371/journal.ppat.1005924
 143. Drake JW, Holland JJ. Mutation rates among RNA viruses. *Proc Natl Acad Sci U S A* (1999) 96(24):13910–3. doi:10.1073/pnas.96.24.13910
 144. Simmonds P, Domingo E. Virus evolution. *Curr Opin Virol* (2011) 1(5):410–2. doi:10.1016/j.coviro.2011.10.021
 145. Sevilla N, de la Torre JC. Arenavirus diversity and evolution: quasispecies in vivo. *Curr Top Microbiol Immunol* (2006) 299:315–35.
 146. Hunziker L, Ciurea A, Recher M, Hengartner H, Zinkernagel RM. Public versus personal serotypes of a viral quasispecies. *Proc Natl Acad Sci U S A* (2003) 100(10):6015–20. doi:10.1073/pnas.1031671100
 147. Grande-Perez A, Sierra S, Castro MG, Domingo E, Lowenstein PR. Molecular indeterminism in the transition to error catastrophe: systematic elimination of lymphocytic choriomeningitis virus through mutagenesis does not correlate linearly with large increases in mutant spectrum complexity. *Proc Natl Acad Sci U S A* (2002) 99(20):12938–43. doi:10.1073/pnas.182426999
 148. Sevilla N, Domingo E, de la Torre JC. Contribution of LCMV towards deciphering biology of quasispecies in vivo. *Curr Top Microbiol Immunol* (2002) 263:197–220.

149. von Hahn T, Yoon JC, Alter H, Rice CM, Rehmann B, Balfe P, et al. Hepatitis C virus continuously escapes from neutralizing antibody and T-cell responses during chronic infection in vivo. *Gastroenterology* (2007) 132(2):667–78. doi:10.1053/j.gastro.2006.12.008
150. Logvinoff C, Major ME, Oldach D, Heyward S, Talal A, Balfe P, et al. Neutralizing antibody response during acute and chronic hepatitis C virus infection. *Proc Natl Acad Sci U S A* (2004) 101(27):10149–54. doi:10.1073/pnas.0403519101
151. Gray ES, Moore PL, Choge IA, Decker JM, Bibollet-Ruche F, Li H, et al. Neutralizing antibody responses in acute human immunodeficiency virus type 1 subtype C infection. *J Virol* (2007) 81(12):6187–96. doi:10.1128/JVI.00239-07
152. Moore JP, Cao Y, Ho DD, Koup RA. Development of the anti-gp120 antibody response during seroconversion to human immunodeficiency virus type 1. *J Virol* (1994) 68(8):5142–55.
153. Zhao J, Nie J, Jiao Y, Li L, Zhang T, Liu Q, et al. Effect of the maturation of neutralizing antibodies on human immunodeficiency virus (HIV) envelope evolution in HIV-infected subjects. *Infect Genet Evol* (2016) 38:82–9. doi:10.1016/j.meegid.2015.12.012
154. Wei X, Decker JM, Wang S, Hui H, Kappes JC, Wu X, et al. Antibody neutralization and escape by HIV-1. *Nature* (2003) 422(6929):307–12. doi:10.1038/nature01470
155. Rong R, Li B, Lynch RM, Haaland RE, Murphy MK, Mulenga J, et al. Escape from autologous neutralizing antibodies in acute/early subtype C HIV-1 infection requires multiple pathways. *PLoS Pathog* (2009) 5(9):e1000594. doi:10.1371/journal.ppat.1000594
156. Ogata N, Alter HJ, Miller RH, Purcell RH. Nucleotide sequence and mutation rate of the H strain of hepatitis C virus. *Proc Natl Acad Sci U S A* (1991) 88(8):3392–6. doi:10.1073/pnas.88.8.3392
157. Farci P, Shimoda A, Wong D, Cabezon T, De Giannis D, Strazzer A, et al. Prevention of hepatitis C virus infection in chimpanzees by hyperimmune serum against the hypervariable region 1 of the envelope 2 protein. *Proc Natl Acad Sci U S A* (1996) 93(26):15394–9. doi:10.1073/pnas.93.26.15394
158. Leonard CK, Spellman MW, Riddle L, Harris RJ, Thomas JN, Gregory TJ. Assignment of intrachain disulfide bonds and characterization of potential glycosylation sites of the type 1 recombinant human immunodeficiency virus envelope glycoprotein (gp120) expressed in Chinese hamster ovary cells. *J Biol Chem* (1990) 265(18):10373–82.
159. Helle F, Duverlie G, Dubuisson J. The hepatitis C virus glycan shield and evasion of the humoral immune response. *Viruses* (2011) 3(10):1909–32. doi:10.3390/v3101909
160. Helle F, Vieyres G, Elkrief L, Popescu C-I, Wychowski C, Descamps V, et al. Role of N-linked glycans in the functions of hepatitis C virus envelope proteins incorporated into infectious virions. *J Virol* (2010) 84(22):11905–15. doi:10.1128/JVI.01548-10
161. Sommerstein R, Flatz L, Remy MM, Malinge P, Magistrelli G, Fischer N, et al. Arenavirus glycan shield promotes neutralizing antibody evasion and protracted infection. *PLoS Pathog* (2015) 11(11):e1005276. doi:10.1371/journal.ppat.1005276
162. Bonhomme CJ, Capul AA, Lauron EJ, Bederka LH, Knopp KA, Buchmeier MJ. Glycosylation modulates arenavirus glycoprotein expression and function. *Virology* (2011) 409(2):223–33. doi:10.1016/j.virol.2010.10.011
163. Binley JM, Wyatt R, Desjardins E, Kwong PD, Hendrickson W, Moore JP, et al. Analysis of the interaction of antibodies with a conserved, enzymatically deglycosylated core of the HIV type 1 envelope glycoprotein 120. *AIDS Res Hum Retrovirus* (1998) 14(3):191–8. doi:10.1089/aid.1998.14.191
164. Koch M, Pancera M, Kwong PD, Kolchinsky P, Grundner C, Wang L, et al. Structure-based, targeted deglycosylation of HIV-1 gp120 and effects on neutralization sensitivity and antibody recognition. *Virology* (2003) 313(2):387–400. doi:10.1016/S0042-6822(03)00294-0
165. Ma B-J, Alam SM, Go EP, Lu X, Desaire H, Tomaras GD, et al. Envelope deglycosylation enhances antigenicity of HIV-1 gp41 epitopes for both broad neutralizing antibodies and their unmutated ancestor antibodies. *PLoS Pathog* (2011) 7(9):e1002200. doi:10.1371/journal.ppat.1002200
166. Qi Y, Jo S, Im W. Roles of glycans in interactions between gp120 and HIV broadly neutralizing antibodies. *Glycobiology* (2016) 26(3):251–60. doi:10.1093/glycob/cwv101
167. McLellan JS, Pancera M, Carrico C, Gorman J, Julien J-P, Khayat R, et al. Structure of HIV-1 gp120 V1/V2 domain with broadly neutralizing antibody PG9. *Nature* (2011) 480(7377):336–43. doi:10.1038/nature10696
168. Pejchal R, Doores KJ, Walker LM, Khayat R, Huang P-S, Wang S-K, et al. A potent and broad neutralizing antibody recognizes and penetrates the HIV glycan shield. *Science* (2011) 334(6059):1097–103. doi:10.1126/science.1213256
169. Kong L, Lee JH, Doores KJ, Murin CD, Julien J-P, McBride R, et al. Supersite of immune vulnerability on the glycosylated face of HIV-1 envelope glycoprotein gp120. *Nat Struct Mol Biol* (2013) 20(7):796–803. doi:10.1038/nsmb.2594
170. Pancera M, Shahzad-ul-Hussan S, Doria-Rose NA, McLellan JS, Bailer RT, Dai K, et al. Structural basis for diverse N-glycan recognition by HIV-1-neutralizing V1–V2-directed antibody PG16. *Nat Struct Mol Biol* (2013) 20(7):804–13. doi:10.1038/nsmb.2600
171. Garces F, Sok D, Kong L, McBride R, Kim HJ, Saye-Francisco KF, et al. Structural evolution of glycan recognition by a family of potent HIV antibodies. *Cell* (2014) 159(1):69–79. doi:10.1016/j.cell.2014.09.009
172. Doria-Rose NA, Klein RM, Manion MM, O'Dell S, Phogat A, Chakrabarti B, et al. Frequency and phenotype of human immunodeficiency virus envelope-specific b cells from patients with broadly cross-neutralizing antibodies. *J Virol* (2009) 83(1):188–99. doi:10.1128/JVI.01583-08
173. Sather DN, Armann J, Ching LK, Mavrantoni A, Sellhorn G, Caldwell Z, et al. Factors associated with the development of cross-reactive neutralizing antibodies during human immunodeficiency virus type 1 infection. *J Virol* (2009) 83(2):757–69. doi:10.1128/JVI.02036-08
174. Simek MD, Rida W, Priddy FH, Pung P, Carrow E, Laufer DS, et al. Human immunodeficiency virus type 1 elite neutralizers: individuals with broad and potent neutralizing activity identified by using a high-throughput neutralization assay together with an analytical selection algorithm. *J Virol* (2009) 83(14):7337–48. doi:10.1128/JVI.00110-09
175. West AP Jr, Scharf L, Scheid JF, Klein F, Bjorkman PJ, Nussenzweig MC. Nussenzweig: structural insights on the role of antibodies in HIV-1 vaccine and therapy. *Cell* (2014) 156(4):633–48. doi:10.1016/j.cell.2014.01.052
176. Walker LM, Huber M, Doores KJ, Falkowska E, Pejchal R, Julien J-P, et al. Broad neutralization coverage of HIV by multiple highly potent antibodies. *Nature* (2011) 477(7365):466–70. doi:10.1038/nature10373
177. Scheid JF, Mouquet H, Ueberheide B, Diskin R, Klein F, Oliveira TYK, et al. Sequence and structural convergence of broad and potent HIV antibodies that mimic CD4 binding. *Science* (2011) 333(6049):1633–7. doi:10.1126/science.1207227
178. Verkoczy L, Kelsoe G, Moody MA, Haynes BF. Role of immune mechanisms in induction of HIV-1 broadly neutralizing antibodies. *Curr Opin Immunol* (2011) 23(3):383–90. doi:10.1016/j.coi.2011.04.003
179. Briney BS, Willis JR, Crowe JE Jr. Human peripheral blood antibodies with long HCDR3s are established primarily at original recombination using a limited subset of germline genes. *PLoS One* (2012) 7(5):e36750. doi:10.1371/journal.pone.0036750
180. Burton DR, Ahmed R, Barouch DH, Butera ST, Crotty S, Godzik A, et al. A blueprint for HIV vaccine discovery. *Cell Host Microbe* (2012) 12(4):396–407. doi:10.1016/j.chom.2012.09.008
181. Wu X, Zhang Z, Schramm CA, Joyce MG, Kwon YD, Zhou T, et al. Maturation and diversity of the VRC01-antibody lineage over 15 years of chronic HIV-1 infection. *Cell* (2015) 161(3):470–85. doi:10.1016/j.cell.2015.03.004
182. Fera D, Schmidt AG, Haynes BF, Gao F, Liao H-X, Kepler TB, et al. Affinity maturation in an HIV broadly neutralizing B-cell lineage through reorientation of variable domains. *Proc Natl Acad Sci U S A* (2014) 111(28):10275–80. doi:10.1073/pnas.1409954111
183. Doria-Rose NA, Schramm CA, Gorman J, Moore PL, Bhiman JN, DeKosky BJ, et al. Developmental pathway for potent V1V2-directed HIV-neutralizing antibodies. *Nature* (2014) 509(7498):55–62. doi:10.1038/nature13036
184. Liao H-X, Lynch R, Zhou T, Gao F, Alam SM, Boyd SD, et al. Co-evolution of a broadly neutralizing HIV-1 antibody and founder virus. *Nature* (2013) 496(7446):469–76. doi:10.1038/nature12053
185. Wibmer CK, Bhiman JN, Gray ES, Tumba N, Abdool Karim SS, Williamson C, et al. Viral escape from HIV-1 neutralizing antibodies drives increased plasma neutralization breadth through sequential recognition of multiple epitopes and immunotypes. *PLoS Pathog* (2013) 9(10):e1003738. doi:10.1371/journal.ppat.1003738

186. Sather DN, Carbonetti S, Malherbe DC, Pissani F, Stuart AB, Hessel AJ, et al. Emergence of broadly neutralizing antibodies and viral coevolution in two subjects during the early stages of infection with human immunodeficiency virus type 1. *J Virol* (2014) 88(22):12968–81. doi:10.1128/JVI.01816-14
187. Gao F, Bonsignori M, Liao H-X, Kumar A, Xia S-M, Lu X, et al. Haynes: cooperation of B cell lineages in induction of HIV-1-broadly neutralizing antibodies. *Cell* (2014) 158(3):481–91. doi:10.1016/j.cell.2014.06.022
188. Rusert P, Kouyos RD, Kadelka C, Ebner H, Schanz M, Huber M, et al. Determinants of HIV-1 broadly neutralizing antibody induction. *Nat Med* (2016) 22(11):1260–7. doi:10.1038/nm.4187
189. Smith SA, Burton SL, Kilembe W, Lakhi S, Karita E, Price M, et al. Diversification in the HIV-1 envelope hyper-variable domains V2, V4, and V5 and higher probability of transmitted/founder envelope glycosylation favor the development of heterologous neutralization breadth. *PLoS Pathog* (2016) 12(11):e1005989. doi:10.1371/journal.ppat.1005989
190. Piantadosi A, Chohan B, Panteleeff D, Baeten JM, Mandalia K, Ndinya-Achola JO, et al. HIV-1 evolution in gag and env is highly correlated but exhibits different relationships with viral load and the immune response. *AIDS* (2009) 23(5):579–87. doi:10.1097/QAD.0b013e328328f76e
191. Doria-Rose NA, Klein RM, Daniels MG, O'Dell S, Nason M, Lapedes A, et al. Breadth of human immunodeficiency virus-specific neutralizing activity in sera: clustering analysis and association with clinical variables. *J Virol* (2010) 84(3):1631–6. doi:10.1128/JVI.01482-09
192. Euler Z, van den Kerkhof TLGM, van Gils MJ, Burger JA, Edo-Matas D, Phung P, et al. Longitudinal analysis of early HIV-1-specific neutralizing activity in an elite neutralizer and in five patients who developed cross-reactive neutralizing activity. *J Virol* (2012) 86(4):2045–55. doi:10.1128/JVI.06091-11
193. Carter CC, Wagner GA, Hightower GK, Caballero G, Phung P, Richman DD, et al. HIV-1 neutralizing antibody response and viral genetic diversity characterized with next generation sequencing. *Virology* (2015) 474:34–40. doi:10.1016/j.virol.2014.10.019
194. Murugan R, Buchauer L, Triller G, Kreschel C, Costa G, Pidelaserra Marti G, et al. Clonal selection drives protective memory B cell responses in controlled human malaria infection. *Sci Immunol* (2018) 3(20):ea8029. doi:10.1126/sciimmunol.aap8029
195. Heesters BA, Lindqvist M, Vagefi PA, Scully EP, Schildberg FA, Altfeld M, et al. Follicular dendritic cells retain infectious HIV in cycling endosomes. *PLoS Pathog* (2015) 11(12):e1005285. doi:10.1371/journal.ppat.1005285
196. Keele BF, Tazi L, Gartner S, Liu Y, Burgon TB, Estes JD, et al. Characterization of the follicular dendritic cell reservoir of human immunodeficiency virus type 1. *J Virol* (2008) 82(11):5548–61. doi:10.1128/JVI.00124-08
197. Alter G, Barouch DH. Natural evolution of broadly neutralizing antibodies. *Cell* (2015) 161(3):427–8. doi:10.1016/j.cell.2015.04.007
198. Gitlin AD, Shulman Z, Nussenzweig MC. Clonal selection in the germinal centre by regulated proliferation and hypermutation. *Nature* (2014) 509(7502):637–40. doi:10.1038/nature13300
199. Lane HC, Masur H, Edgar LC, Whalen G, Rook AH, Fauci AS. Abnormalities of B-cell activation and immunoregulation in patients with the acquired immunodeficiency syndrome. *N Engl J Med* (1983) 309(8):453–8. doi:10.1056/NEJM198308253090803
200. Moir S, Fauci AS. B cells in HIV infection and disease. *Nat Rev Immunol* (2009) 9(4):235–45. doi:10.1038/nri2524
201. Meyer K, Ait-Goughoulte M, Keck Z-Y, Fong S, Ray R. Antibody-dependent enhancement of hepatitis C virus infection. *J Virol* (2008) 82(5):2140–9. doi:10.1128/JVI.01867-07
202. Hunziker L, Recher M, Macpherson AJ, Ciurea A, Freigang S, Hengartner H, et al. Hypergammaglobulinemia and autoantibody induction mechanisms in viral infections. *Nat Immunol* (2003) 4(4):343–9. doi:10.1038/ni911
203. Shirai A, Cosentino M, Leitman-Klinman SE, Klinman DM. Human immunodeficiency virus infection induces both polyclonal and virus-specific B cell activation. *J Clin Invest* (1992) 89(2):561–6. doi:10.1172/JCI115621
204. Rosa D, Saletti G, De Gregorio E, Zorat F, Comar C, D'Oro U, et al. Activation of naïve B lymphocytes via CD81, a pathogenetic mechanism for hepatitis C virus-associated B lymphocyte disorders. *Proc Natl Acad Sci U S A* (2005) 102(51):18544–9. doi:10.1073/pnas.0509402102
205. Lei Y, Hu T, Song X, Nie H, Chen M, Chen W, et al. Production of autoantibodies in chronic hepatitis B virus infection is associated with the augmented function of blood CXCR5+CD4+ T cells. *PLoS One* (2016) 11(9):e0162241. doi:10.1371/journal.pone.0162241
206. Di Niro R, Lee SJ, Vander Heiden JA, Elsner RA, Trivedi N, Bannock JM, et al. Shlomchik: *Salmonella* infection drives promiscuous B cell activation followed by extrafollicular affinity maturation. *Immunity* (2015) 43(1):120–31. doi:10.1016/j.immuni.2015.06.013
207. Jellison ER, Guay HM, Szomolanyi-Tsuda E, Welsh RM. Dynamics and magnitude of virus-induced polyclonal B cell activation mediated by BCR-independent presentation of viral antigen. *Eur J Immunol* (2007) 37(1):19–28. doi:10.1002/eji.200636516
208. Sanderson NSR, Zimmermann M, Eilinger L, Gubser C, Schaeren-Wiemers N, Lindberg RLP, et al. Cocapture of cognate and bystander antigens can activate autoreactive B cells. *Proc Natl Acad Sci U S A* (2017) 114(4):734–9. doi:10.1073/pnas.1614472114
209. Crotty S. T follicular helper cell differentiation, function, and roles in disease. *Immunity* (2014) 41(4):529–42. doi:10.1016/j.immuni.2014.10.004
210. Simpson N, Gatenby PA, Wilson A, Malik S, Fulcher DA, Tangye SG, et al. Expansion of circulating T cells resembling follicular helper T cells is a fixed phenotype that identifies a subset of severe systemic lupus erythematosus. *Arthritis Rheum* (2010) 62(1):234–44. doi:10.1002/art.25032
211. Ma J, Zhu C, Ma B, Tian J, Baidoo SE, Mao C, et al. Increased frequency of circulating follicular helper T cells in patients with rheumatoid arthritis. *Clin Dev Immunol* (2012) 2012:827480. doi:10.1155/2012/827480
212. Szabo K, Papp G, Barath S, Gyimesi E, Szanto A, Zeher M. Follicular helper T cells may play an important role in the severity of primary Sjögren's syndrome. *Clin Immunol* (2013) 147(2):95–104. doi:10.1016/j.clim.2013.02.024
213. Szabó K, Papp G, Szántó A, Tarr T, Zeher M. A comprehensive investigation on the distribution of circulating follicular T helper cells and B cell subsets in primary Sjögren's syndrome and systemic lupus erythematosus. *Clin Exp Immunol* (2016) 183(1):76–89. doi:10.1111/cei.12703
214. Pratama A, Vinuesa CG. Control of T_{FH} cell numbers: why and how? *Immunol Cell Biol* (2014) 92(1):40–8. doi:10.1038/icb.2013.69
215. Hong JJ, Amancha PK, Rogers K, Ansari AA, Villinger F. Spatial alterations between CD4+ T follicular helper, B, and CD8+ T cells during simian immunodeficiency virus infection: T/B cell homeostasis, activation, and potential mechanism for viral escape. *J Immunol* (2012) 188(7):3247–56. doi:10.4049/jimmunol.1103138

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2018 Greczmiel and Oxenius. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



Relapsing–Remitting Multiple Sclerosis Is Characterized by a T Follicular Cell Pro-Inflammatory Shift, Reverted by Dimethyl Fumarate Treatment

Vanesa Cunill^{1,2}, Margarita Massot³, Antonio Clemente^{2,4}, Carmen Calles³, Valero Andreu², Vanessa Núñez³, Antonio López-Gómez^{1,2}, Rosa María Díaz³, María de los Reyes Jiménez^{1,2}, Jaime Pons^{1,2}, Cristòfol Vives-Bauzá⁵ and Joana Maria Ferrer^{1,2*}

¹Immunology Department, Hospital Universitari Son Espases, Palma, Spain, ²Human Immunopathology Research Laboratory, Institut d'Investigació Sanitària de les Illes Balears (IdISBa), Palma, Spain, ³Neurology Department, Hospital Universitari Son Espases, Palma, Spain, ⁴Clinical Trials and Methodology Support Platform, Institut d'Investigació Sanitària de les Illes Balears (IdISBa), Palma, Spain, ⁵Research Unit, Institut d'Investigació Sanitària de les Illes Balears and Hospital Universitari Son Espases, Palma, Spain

OPEN ACCESS

Edited by:

Georgia Fousteri,
San Raffaele Hospital (IRCCS), Italy

Reviewed by:

Hu Zeng,
Mayo Clinic, United States
Xing Chang,
Shanghai Institutes for Biological
Sciences (CAS), China

*Correspondence:

Joana Maria Ferrer
juanam.ferrer@ssib.es

Specialty section:

This article was submitted to
T Cell Biology,
a section of the journal
Frontiers in Immunology

Received: 08 February 2018

Accepted: 02 May 2018

Published: 29 May 2018

Citation:

Cunill V, Massot M, Clemente A, Calles C, Andreu V, Núñez V, López-Gómez A, Díaz RM, Jiménez MdR, Pons J, Vives-Bauzá C and Ferrer JM (2018) Relapsing–Remitting Multiple Sclerosis Is Characterized by a T Follicular Cell Pro-Inflammatory Shift, Reverted by Dimethyl Fumarate Treatment. *Front. Immunol.* 9:1097. doi: 10.3389/fimmu.2018.01097

Multiple sclerosis (MS) is considered a T cell-mediated autoimmune disease, although several evidences also demonstrate a B cell involvement in its etiology. Follicular T helper (Tfh) cells, a CXCR5-expressing CD4+ T cell subpopulation, are essential in the regulation of B cell differentiation and maintenance of humoral immunity. Alterations in circulating (c)Tfh distribution and/or function have been associated with autoimmune diseases including MS. Dimethyl fumarate (DMF) is a recently approved first-line treatment for relapsing–remitting MS (RRMS) patients whose mechanism of action is not completely understood. The aim of our study was to compare cTfh subpopulations between RRMS patients and healthy subjects and evaluate the impact of DMF treatment on these subpopulations, relating them to changes in B cells and humoral response. We analyzed, by flow cytometry, the distribution of cTfh1 (CXCR3+CCR6–), cTfh2 (CXCR3–CCR6–), cTfh17 (CXCR3–CCR6+), and the recently described cTfh17.1 (CXCR3+CCR6+) subpopulations of CD4+ Tfh (CD45RA–CXCR5+) cells in a cohort of 29 untreated RRMS compared to healthy subjects. CD4+ non-follicular T helper (Th) cells (CD45RA–CXCR5–) were also studied. We also evaluated the effect of DMF treatment on these subpopulations after 6 and 12 months treatment. Untreated RRMS patients presented higher percentages of cTfh17.1 cells and lower percentages of cTfh2 cells consistent with a pro-inflammatory bias compared to healthy subjects. DMF treatment induced a progressive increase in cTfh2 cells, accompanied by a decrease in cTfh1 and the pathogenic cTfh17.1 cells. A similar decrease of non-follicular Th1 and Th17.1 cells in addition to an increase in the anti-inflammatory Th2 subpopulation were also detected upon DMF treatment, accompanied by an increase in naïve B cells and a decrease in switched memory B cells and serum levels of IgA, IgG2, and IgG3. Interestingly, this effect was not observed in three patients in whom DMF had to be discontinued due to

an absence of clinical response. Our results demonstrate a possibly pathogenic cTfh pro-inflammatory profile in RRMS patients, defined by high cTfh17.1 and low cTfh2 subpopulations that is reverted by DMF treatment. Monitoring cTfh subsets during treatment may become a biological marker of DMF effectiveness.

Keywords: multiple sclerosis, dimethyl fumarate, follicular T cells, cTfh17.1, B cells

INTRODUCTION

Multiple sclerosis (MS), one of the most common causes of neurological disability in young adults, is a chronic progressive neurodegenerative autoimmune disease of the central nervous system (CNS), which leads to inflammation, demyelination, and axonal damage in the brain and spinal cord (1). Based on symptoms onset and clinical course, two main types of MS can be described: relapsing–remitting MS (RRMS) and progressive MS. RRMS affects 85% MS patients and is characterized by young adulthood onset episodes of acute exacerbations followed by complete or partial recovery. Many of these patients develop a secondary progressive form of MS (PSMS) with gradual progression of disability. The annual conversion rate into PSMS is 2.5%, approximately. Primarily progressive MS, characterized by continuous worsening without relapses, accounts for only 15% of MS patients (2).

Multiple sclerosis etiology is still incompletely understood. Both CD4+ and CD8+ T cells and B cells have been described as important players in MS pathogenesis. Historically, autoreactive IFN γ -producing T helper (Th) 1 cells were considered the main mediators of inflammation causing MS lesions (3). This model was challenged with the discovery of interleukin (IL)-17-producing Th17 cells. Th17 cells secrete pro-inflammatory cytokines (IL-17 and IL-6) and express CCR6, the CCL20 ligand expressed on vascular endothelial cells that allows them to pass through the blood–brain barrier (4). However, although MS is thought to be a T cell-mediated disease, several lines of evidence demonstrate the involvement of B cells in disease course. The presence of oligoclonal bands in cerebrospinal fluid in as many as 95% diagnosed patients and the presence of B cells, plasma cells, and meningeal B-cell follicles in the CNS, point to the involvement of B cells and antibody production in MS (5). Moreover, clinical trials using B cell-depleting therapies suggests that a decrease in B cell antigen presenting ability and a change in B cells cytokine production contribute to reduce MS activity (6).

Significant advances in the development of disease-modifying drugs for RRMS have been achieved. Dimethyl fumarate (DMF) is an oral fumaric acid ester approved by the FDA and EMA in 2013 as a first-line treatment for RRMS. Clinical trials of DMF-treated RRMS patients showed significant reductions in clinical relapses and MRI evidence of inflammatory disease (7). The mechanism of action of DMF is not completely understood, but it is known that DMF reduces oxidative stress and modulates nuclear factor kappa B, which could have anti-inflammatory effects, mainly mediated through the activation of the Nrf2 pathway (8). Recent studies have shown a reduction of CD4+ T, CD8+ T, B, and NKT cells, and a shift in Th subpopulations in RRMS patients treated with DMF (9).

The follicular T helper (Tfh) cells are a CD4+ T cell subpopulation essential in the regulation of humoral immunity, specialized in supporting B cell maturation and immunoglobulin production in secondary lymphoid organs. Tfh cells were first described as a CXCR5-expressing population localizing in “tonsillar” follicles (10, 11). The discovery of a circulating counterpart of this population has allowed investigating their relevance in health and disease (12). Morita et al. originally demonstrated that analogous to non-follicular Th cells, circulating Tfh (cTfh) cells can be classified, according to the expression of CXCR3 and CCR6, into cTfh1 (CXCR3+CCR6–), cTfh2 (CXCR3–CCR6–), and cTfh17 (CXCR3–CCR6+) whose differentiation relies on distinct transcription factors (12). These subpopulations produce a different set of cytokines and exert different B helper cell capabilities. Alterations in cTfh function and/or distribution have been associated with the pathogenesis of many autoimmune diseases, including MS (13, 14) and also with infectious diseases and monogenic immunodeficiencies (15). A CXCR3+CCR6+ Th subpopulation that rivals with Th1 in IFN γ production, but also produces IL-17, has been described as Th17.1 and identified in sarcoidosis and Crohn’s disease (16–18). A follicular CXCR5-expressing counterpart (cTfh17.1) has also been recently identified in common variable immunodeficiency (19).

The aim of the present study was to compare cTfh subpopulations between RRMS patients and healthy controls, to evaluate the impact of DMF treatment on these subpopulations and its relationship with changes in B cell distribution and clinical evolution.

MATERIALS AND METHODS

Patients

29 patients, 19 females, and 10 males, diagnosed with RRMS defined by 2010 McDonald criteria (20) were included in the study. All RRMS patients were treated with 240 mg DMF twice a day and followed 6 and 12 months after starting treatment. 35 age- and sex-matched healthy subjects were included as controls.

Patients mean age was 41.2 years (range 26–60). Mean time since first MS appearing symptoms was 6.1 ± 5.1 years. Clinical relapses were evaluated in our cohort before DMF treatment. The number of clinical relapses 1 and 2 years previous to DMF treatment was (0.58 ± 0.5) and (0.79 ± 0.6) , respectively. 15 patients had ≥ 1 gadolinium-enhancing (Gd+) lesions, 22 had >10 supratentorial T2*-weighted lesions, and 18 >1 infratentorial T2*-weighted lesions in baseline MRI findings.

The mean Expanded Disability Status Scale (EDSS) was 1.8 ± 1.3 prior to DMF treatment, 1.8 ± 1.2 after 6 months

treatment, and 2.1 ± 1.3 after 12 months treatment, the change not proving significant (paired test; $p = 0.82$). 10 patients diagnosed with RRMS had not received any previous treatment and 20 had received first line drugs (14 interferon- β and 5 glatiramer-acetate) before starting DMF treatment.

The 14 patients included in the longitudinal cohort displayed similar demographic and clinical characteristics.

The study was conducted according to the ethical guidelines of the 1975 Declaration of Helsinki and approved by CEIC (Balearic Islands Clinical Research Ethics Committee). Written informed consent was obtained from all subjects.

Flow Cytometry

Cell surface markers expression was analyzed by flow cytometry using a Navios cytometer and data evaluated using Kaluza software (Beckman Coulter, USA).

A surface staining protocol was performed to analyze membrane antigen expression in lymphocytes subpopulations. Briefly, 100 μ L of peripheral fresh whole blood were incubated 20 min at room temperature (25°C) with different fluorochrome-conjugated monoclonal antibodies combinations. Red blood cells were lysed, white cells fixed using TQ-Prep System (Beckman Coulter), and 100 μ L of fluorospheres Flow-Count™ (Fluorospheres, Beckman Coulter) were added to calculate cell concentration, previous to flow cytometric analysis.

Combinations of the following antibodies were used: anti-CD45-FITC, anti-CD4-PE, anti-CD8-ECD, anti-CD3-PCy5, anti-CD56-PCy5, anti-CD3-PCy7, and anti-CD45RA-ECD (all from Coulter Immunotech, France) to evaluate T and NK cells; anti-CD19-ECD, anti-CD27-PCy7, anti-CD21-FITC, anti-CD24-PCy5, anti-CD38-PCy7 (all from Coulter Immunotech, France) and anti-IgD-FITC (Dako, Spain) to study B cells; anti-CD4-PCy5, anti-CD45RA-ECD (from Coulter Immunotech, France), anti-CXCR5-PE (R&D Systems, Spain), anti-CXCR3-FITC and anti-CCR6-PCy7 (Biolegend, USA) to evaluate Th and cTfh subpopulations.

Nephelometry

Serum total IgG, IgA, IgM levels quantification and IgG subclass profile (IgG1, IgG2, IgG3, IgG4) were assayed by nephelometry in a BN II nephelometer (Siemens, Germany). Commercially available kits (Siemens, Germany) were used according to manufacturer's instructions protocol. The intra and inter-assay coefficients of variations were less than 5%.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism version 4.0 software. Data are expressed as mean values. The Mann-Whitney *U*-test was used to compare differences between controls and untreated RRMS patients or controls and RRMS 12 months DMF-treated patients. The Kruskal-Wallis test was used to compare differences between subgroups of RRMS patients.

For longitudinal samples, Wilcoxon and Friedman test were used to compare paired data. We used Wilcoxon test to compare differences between untreated and 12 months DMF-treated RRMS patients. Friedman test along with a Dunn multiple comparison test were applied to assess differences between untreated RRMS

patients and 6 and 12 months DMF-treated RRMS patients. A p -value ≤ 0.05 was considered statistically significant.

RESULTS

Peripheral Blood Lymphocyte Populations in Untreated RRMS Patients

Percentages and absolute counts of CD4+ and CD8+ T cells, B, and NK cells in untreated RRMS patients were within reported ranges for Caucasians (21, 22) (Table 1).

However, B cells subsets distribution was altered (Figures 1A,B). When we compared B cells subsets from untreated RRMS patients with those of our healthy subjects' cohort, we observed a trend toward decreased percentages of naïve B cells (CD19+CD27-IgD+) in untreated RRMS patients (52 vs. 60.7%; $p = 0.09$) (Figure 1C). The percentages of transitional (CD19+CD38^{high}CD24^{high}), plasmablasts (CD19+CD38^{high}CD24-) and non-switched memory (CD19+CD27-IgD+) B cells were similar between untreated RRMS patients and controls (Figures 1C-F). However, we found a significantly higher percentage of switched-memory (CD19+CD27-IgD-CD27+) B cells in untreated RRMS patients (25.9 vs. 16.5%; $p < 0.01$) compared to healthy subjects (Figure 1G).

Selectively Increased cTfh17.1 and Decreased cTfh2 Effector Cells in Untreated RRMS Patients

No differences were found in the percentage of total CD4+CXCR5+ cTfh cells between untreated RRMS patients and controls (10.8 vs. 10.8%) (Figures 2A,B). Several studies have shown that cTfh cells are contained within the memory CD45RA-CD4+ T cells. No differences were found in the percentage of circulating cTfh cells, analyzed as memory CD4+CD45RA-CXCR5+, in untreated RRMS patients compared to controls (9.1 vs. 9.3%) (Figure 2C).

We further studied the distribution of different subsets of circulating effector T helper non-follicular (Th) CD4+CD45RA-CXCR5- and follicular (cTfh) CD4+CD45RA-CXCR5+ cells, identified according to the expression of CXCR3 and CCR6 markers in untreated RRMS patients and controls: Th1/cTfh1 (CXCR3+CCR6-), Th2/cTfh2 (CXCR3-CCR6-), Th17/cTfh17 (CXCR3-CCR6+), and Th17.1/cTfh17.1 (CXCR3+CCR6+) (Figure 2D).

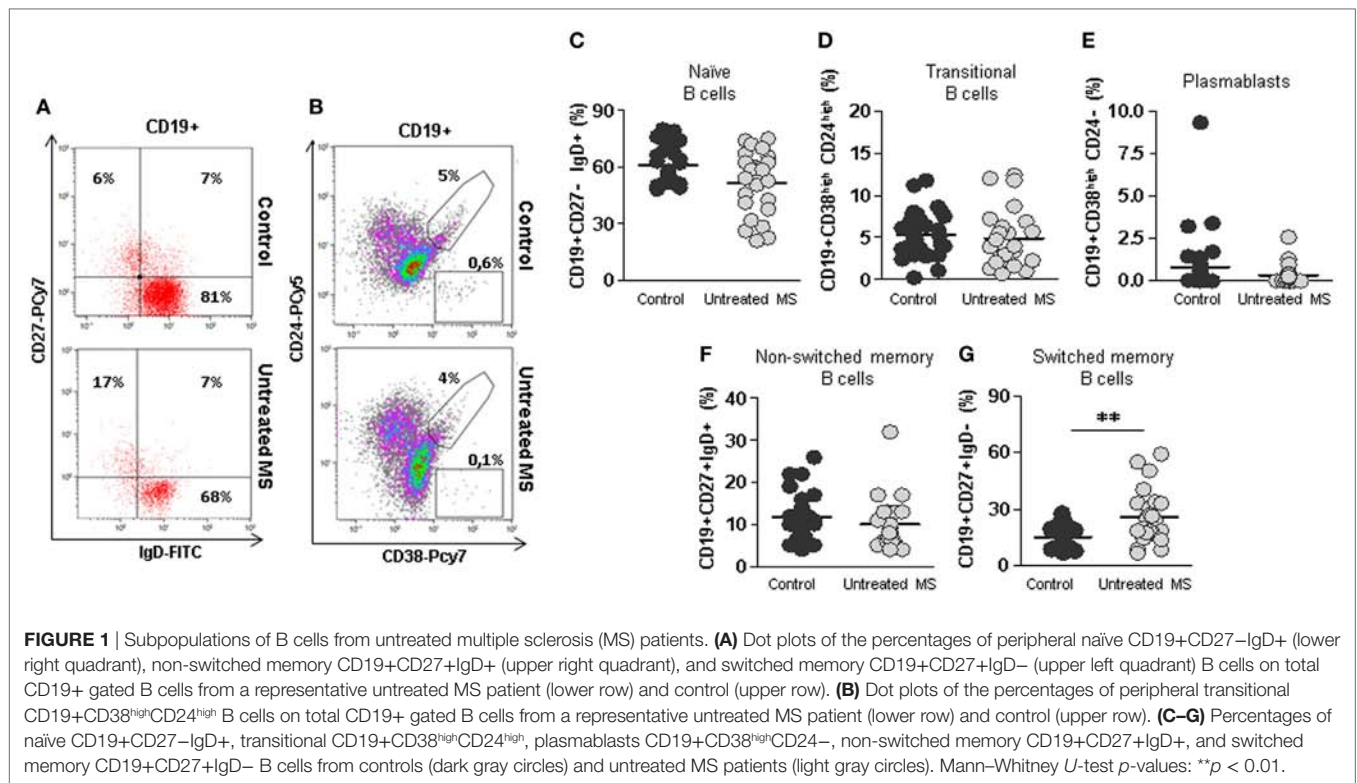
No differences were found when Th1, Th17, and Th2 non-follicular subpopulations were compared (Figures 2E-G). There was a trend toward higher percentage of Th17.1 cells in untreated RRMS patients, although it was not statistically significant (Figure 2H). Interestingly, when cTfh1, cTfh17, and cTfh2 CD4+CD45RA-CXCR5+ subpopulations were evaluated, we found a lower percentage of cTfh2 cells in untreated RRMS patients compared to controls (37.4 vs. 49.9%; $p < 0.001$) (Figure 2K), whereas the percentage of cTfh17.1 was significantly increased (10.0 vs. 4.2%; $p < 0.001$) (Figure 2L). No differences were found in cTfh1 and cTfh17 subpopulations (Figures 2I,J).

To rule out the possibility that differences found in switched-memory B cells or cTfh17.1 and cTfh2 subpopulations were caused by previous treatments, these subpopulations were

TABLE 1 | Lymphocytes subpopulations of untreated multiple sclerosis (MS) patients.

Patients	CD3+ %	CD4+ %	CD4+ cells/ μ L	CD8+ %	CD8+ cells/ μ L	CD19+ %	CD19+ cells/ μ L	CD3– CD56+ %	CD3– CD56+ cells/ μ L	CD19+ (%)					
										CD21 ^{low}	IgD+ CD27–	IgD+ CD27+	IgD– CD27+	CD38 ^{high} CD24 ^{high}	CD38 ^{high} CD24–
1	66	45	908	21	433	16	323	16	323	10	48	32	17	1.3	0.3
2	71	50	641	19	250	15	197	10	132	4	75	6	9	3.39	0.1
3	66	50	1,105	14	318	23	497	9	199	1.3	74	9	9	12	0
4	87	57	738	28	358	5	69	7	94	NA	NA	NA	NA	NA	NA
5	77	45	803	31	378	11	197	11	197	6	67	5	15	NA	NA
6	74	56	820	16	237	14	210	12	170	3	60	5	27	2.85	0
7	78	62	953	14	218	11	162	8	126	9	26	8	50	1.03	0.5
8	83	45	742	36	729	11	182	4	72	8	43	13	33	3.89	0
9	73	49	1,353	22	578	16	439	11	315	19	51	13	24	2.2	0.1
10	72	45	892	22	458	16	313	10	193	7	61	9	19	5.53	0
11	65	42	729	20	338	5	94	26	454	10	59	6	25	3.3	0
12	76	51	509	22	209	15	152	8	77	7	59	6	18	6.89	0.2
13	74	45	485	27	269	16	169	10	102	8	53	4	31	8.7	0
14	68	51	NA	15	NA	21	NA	NA	NA	NA	NA	NA	NA	2.17	1.29
15	76	61	1,136	13	241	13	233	8	140	10	28	6	59	0.7	2.6
16	81	57	1,333	20	558	9	202	7	156	NA	65	8	20	5.72	0
17	66	47	677	18	257	6	88	25	354	19	46	17	27	2.93	0.37
18	73	49	768	19	329	14	219	13	203	7	54	7	27	NA	NA
19	63	46	503	17	181	27	298	8	84	11	72	4	7	12.3	0.1
20	72	55	1,073	17	314	18	348	8	152	4	62	8	19	4	0
21	72	37	533	34	482	16	222	8	109	6	70	10	14	6.27	0.4
22	80	54	924	24	396	9	158	7	114	11	22	8	55	1.61	0.06
23	82	50	1,485	27	844	10	307	7	209	9	58	13	18	5.58	0.15
24	82	52	359	27	182	9	63	6	40	15	28	11	41	2.24	1
25	80	57	1,249	20	417	9	202	8	177	10	42	17	34	4.72	0.44
26	70	43	NA	21	NA	12	NA	10	NA	10	45	13	33	7.32	0.3
27	79	63	NA	14	NA	9	NA	9	NA	6	54	13	19	3.08	0.43
28	77	47	998	23	486	9	191	17	362	NA	NA	NA	NA	7.2	0
29	87	69	1,091	16	263	10	159	3	47	NA	NA	NA	NA	11.7	0.2
Reference ranges	53–83	30–59	490–1,640	10–40	170–880	5–21	80–490	5–32	80–690	1–8	53–86	3–13	4–22	0.9–6.3	0.1–1.5

T (CD3+, CD3+CD4+ and CD3+CD8+), *NK* (CD3–CD56+), and *B* (CD19+) cells were evaluated as percentages from total lymphocytes and as absolute counts (cells/ μ L). Percentages of CD21^{low}, naïve IgD+CD27–, non-switched memory IgD+CD27+, switched memory IgD–CD27+, transitional CD38^{high}CD24^{high} and plasmablasts CD38^{high}CD24– *B* cell subpopulations (referred to total CD19+ *B* lymphocytes) of MS patients. NA, not available.



compared between RRMS patients who had or not received previous disease-modifying treatments. Interestingly, no differences were found in the percentages of switched-memory B, cTfh2 and cTfh17.1 cells (data not shown).

These results demonstrate a clear selective pro-inflammatory shift in cTfh subpopulations and B cells in RRMS patients, not present in the non-follicular Th subpopulations, which is not caused by previous treatments.

DMF Treatment Reduces CD4+ and CD8+ T, NK, and B Cells Counts

We next studied the effect of DMF treatment on the different lymphocyte subpopulations. In order to do so, we compared lymphocyte subpopulations in “short term” 6 months and “long term” 12 months treated RRMS patients with the ones of untreated RRMS patients. We found lower absolute numbers of CD4+ T cells both after 6 and 12 months treatment compared to untreated patients (588.4 vs. 877.2 cells/ μ L; *p* < 0.01) and (555.8 vs. 877.2 cells/ μ L; *p* < 0.01), respectively (Figure 3A). CD8+ T cells counts were lower in 6 months (230.0 vs. 374.0 cells/ μ L; *p* < 0.001) and 12 months treated (223.3 vs. 374.0 cells/ μ L; *p* < 0.001) (Figure 3B) compared to untreated patients. NK and B cells counts were also lower in 6 months treated compared to untreated patients (111.6 vs. 177.0 cells/ μ L and 137.7 vs. 219.0 cells/ μ L; *p* < 0.05 and *p* < 0.01, respectively) (Figures 3C,D). This is consistent with the reported lymphopenia described in DMF-treated RRMS patients (9). In agreement with previous studies (9), we did not find differences in the percentage of any lymphocyte subset between 6 and 12 months treated compared to untreated patients (Figures 3E–H).

DMF Treatment Alters B Cells Distribution and Immunoglobulins Levels

The distribution of B cells subpopulations was significantly altered by DMF treatment (Figures 4A,B). When DMF-treated RRMS patients were compared with untreated patients, we found significantly increased percentages of naïve B cells after 12 months treatment (69.5 vs. 52.9%; *p* < 0.01), transitional B cells after 6 and 12 months treatment (11.4 vs. 4.8% and 12.3 vs. 4.8%; *p* < 0.01, respectively) and plasmablasts after 6 and 12 months treatment (0.8 vs. 0.3% and 1.2 vs. 0.3%; *p* < 0.05 and *p* < 0.001, respectively) (Figures 4C–E). On the contrary, a significant decrease in switched memory B cells was found in 12 months treated compared to untreated RRMS patients (14.3 vs. 25.9%; *p* < 0.01) (Figure 4G). No differences were found in non-switched memory B cells (Figure 4F).

To evaluate if these B cell subpopulation changes had any observable humoral consequence, we quantified immunoglobulin levels in serum samples from 23 longitudinally followed patients before and after 12 months DMF treatment. We found significantly lower levels of IgA in 12 months treated patients compared to untreated patients (Wilcoxon paired test; *p* < 0.01) (Figure 4I). No differences were found when the effect of DMF on IgG and IgM serum levels was evaluated (Figures 4H,J).

We also studied if DMF induced changes in the serum IgG subclass profile (IgG1, IgG2, IgG3 and IgG4). Interestingly, although IgG levels were similar before and after 12 months DMF treatment, the IgG subclass profile was altered. We found significantly lower levels of IgG3 and IgG2 in the serum of 12 months DMF-treated RRMS patients compared to untreated patients (Wilcoxon paired test; *p* < 0.05 and *p* < 0.01, respectively) (Figure 4H).

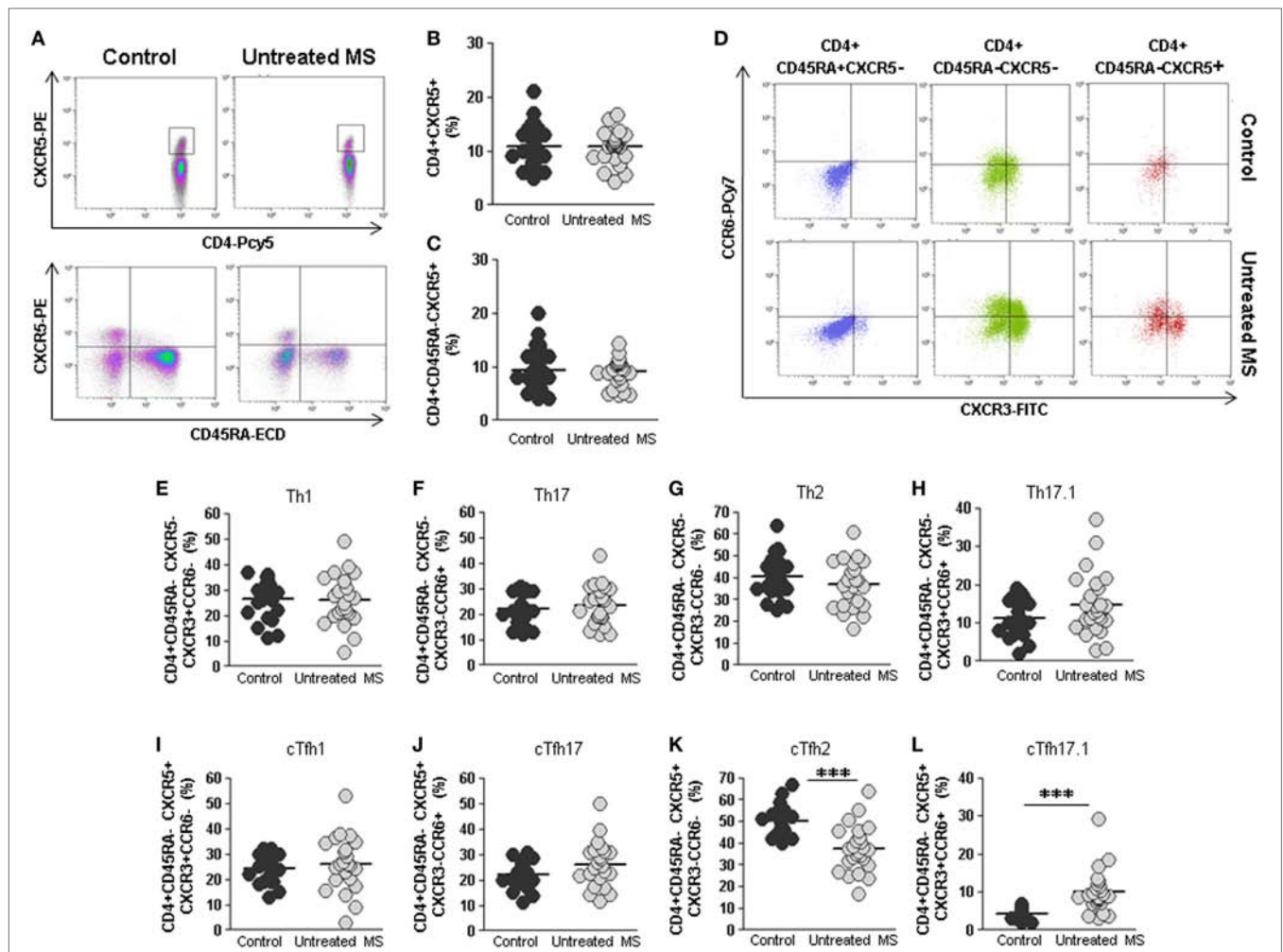


FIGURE 2 | Total cTfh cells and subpopulations of Th and cTfh cells from untreated multiple sclerosis (MS) patients. **(A)** Density plots of the percentage of total follicular CD4+CXCR5+ (upper plots) and memory follicular CD4+CD45RA-CXCR5+ (lower plots) cells from a representative untreated MS patient (right) and control (left). **(B)** Percentages of total follicular CD4+CXCR5+ cells from controls (dark gray circles) and untreated MS patients (light gray circles). **(C)** Percentages of total memory follicular CD4+CD45RA-CXCR5+ cells from healthy controls (dark gray circles) and untreated MS patients (light gray circles). **(D)** Dot-plots of the percentage of Th1 CXCR3+CCR6- (lower right quadrant), Th17 CXCR3-CCR6+ (upper left quadrant), Th2 CXCR3-CCR6- (lower left quadrant), and Th17.1 CXCR3+CCR6+ (upper right quadrant) subpopulations on circulating naïve CD4+CD45RA+CXCR5- (left), non-follicular CD4+CD45RA-CXCR5- (middle), and follicular CD4+CD45RA-CXCR5+ (right) cells from a representative untreated MS patient (lower row) and control (upper row). **(E-H)** Percentages of Th1, Th17, Th2, and Th17.1 non-follicular T cells from controls (dark gray circles) and untreated MS patients (light gray circles). **(I-L)** Percentages of cTfh1, cTfh17, cTfh2, and cTfh17.1 follicular T cells from controls (dark gray circles) and untreated MS patients (light gray circles). Mann-Whitney *U*-test *p*-values: ****p* < 0.001.

DMF Treatment Reduces Both Absolute Numbers and Percentage of cTfh Cells in RRMS Patients

The percentage of CD4+CXCR5+ cTfh cells was lower in 12 months DMF-treated compared to untreated RRMS patients (7.7 vs. 11.4%; *p* < 0.01) (Figure 5A, upper panel). We also found lower cTfh absolute counts in 6 months treated (54.8 vs. 97.0 cells/ μ L; *p* < 0.01) and 12 months (41.8 vs. 97.0 cells/ μ L; *p* < 0.001) compared to untreated RRMS patients (Figure 5A, lower panel).

Lower memory CD4+CD45RA-CXCR5+ cTfh cells percentages and absolute counts were also found in 6 months (7.5 vs.

9.9%; *p* < 0.05 and 43.0 vs. 84.7 cells/ μ L, *p* < 0.001, respectively) and 12 months treated RRMS patients (6.9 vs. 9.9%; *p* < 0.01 and 37.2 vs. 84.7 cells/ μ L *p* < 0.001, respectively) once compared to untreated patients (Figure 5B).

The percentage of non-follicular CD4+CD45RA-CXCR5- Th cells only differed after 6 months DMF treatment (34.1 vs. 47.0%; *p* < 0.05) (Figure 5C, upper panel), although the absolute counts significantly decreased after 6 and 12 months DMF treatment (170.2 vs. 361.8 cells/ μ L and 196.0 vs. 361.8 cells/ μ L; *p* < 0.01 and *p* < 0.05, respectively) (Figure 5C, lower panel). No differences were found when naïve CD4+CD45RA+CXCR5- Th cells subpopulations were compared (data not shown).

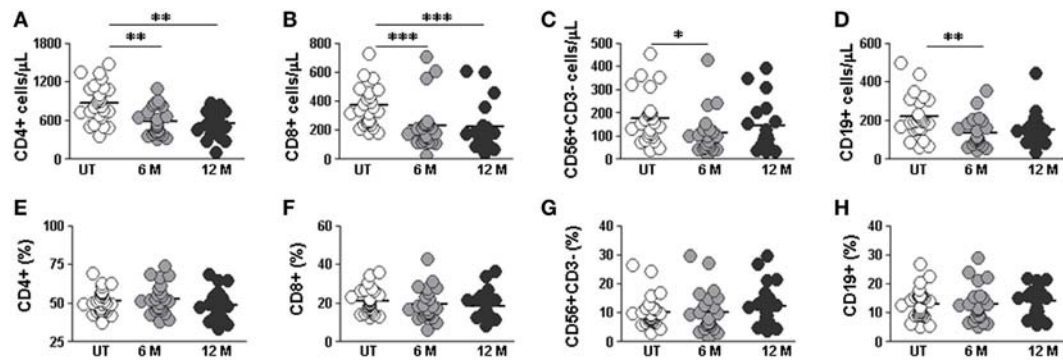


FIGURE 3 | Effect of dimethyl fumarate on CD4, CD8, NK, and B cells from multiple sclerosis (MS) patients. (A–D) Cell counts and (E–H) percentages of peripheral CD3+CD4+, CD3+CD8+, CD56+CD3– (NK), and CD19+ (B) lymphocytes from untreated (UT: white circles), 6 months treated (6 months: light gray circles), and 12 months treated (12 months: dark gray circles) MS patients. Kruskal–Wallis test p -values: * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

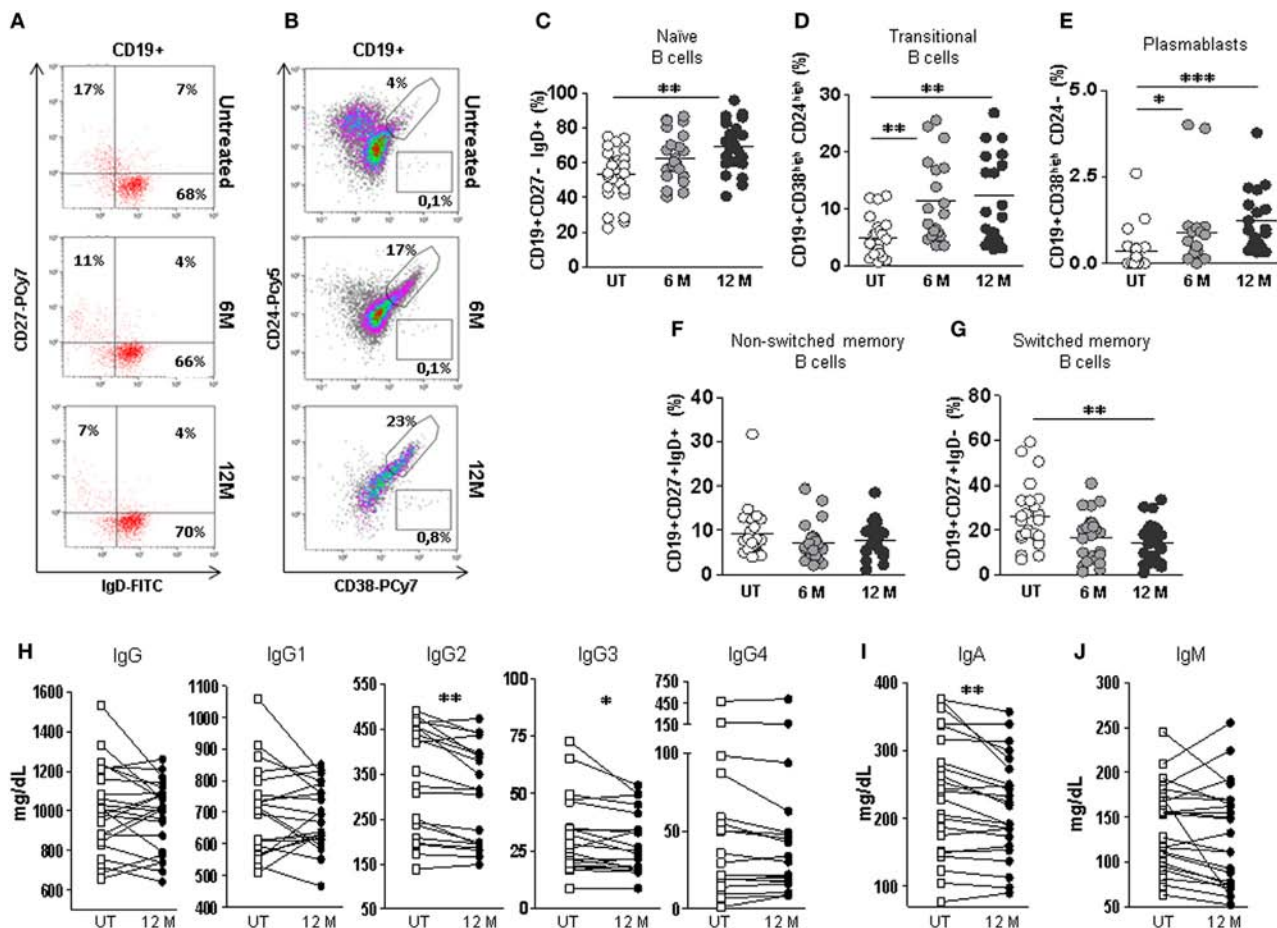


FIGURE 4 | Effect of dimethyl fumarate on B cells subpopulations and serum levels of immunoglobulins in multiple sclerosis (MS) patients. (A) Dot-plots of the percentages of peripheral naïve CD19+CD27–IgD+ (lower right quadrant), non-switched memory CD19+CD27+IgD+ (upper right quadrant), and switched memory CD19+CD27+IgD– (upper left quadrant) B cells on total CD19+ gated B cells from a representative untreated (upper row), 6 months treated (6 M: middle row), and 12 months treated (12 M: lower row) MS patient. (B) Dot plots of the percentages of peripheral transitional CD19+CD38^{high}CD24^{high} B cells on total CD19+ gated B cells from a representative untreated (upper row), 6 months treated (6 M: middle row), and 12 months treated (12 M: lower row) MS patient. (C–G) Percentages of peripheral naïve CD19+CD27–IgD+, transitional CD19+CD38^{high}CD24^{high}, plasmablasts CD19+CD38^{high}CD24–, non-switched memory CD19+CD27+IgD+, and switched memory CD19+CD27+IgD– B cells from untreated (UT: white circles), 6 months treated (6 M: light gray circles), and 12 months treated (12 M: dark gray circles) MS patients. Kruskal–Wallis test p -values: * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. (H–J) Serum IgG, IgG1, IgG2, IgG3, IgG4, IgA, and IgM levels (mg/dL) from MS patients before treatment (UT: white squares) and 12 months (12 M: dark gray circles) after treatment. Successive repeated measures on the same individual are represented by connecting lines. Wilcoxon paired test: p -values: * $p < 0.05$, ** $p < 0.01$.

Increase of cTfh2 and Decrease of cTfh1 and cTfh17.1 Effector Cells in DMF-Treated RRMS Patients

We next compared the effect of DMF on the distribution of non-follicular Th and follicular Tfh effector subpopulations between untreated and 6 and 12 months treated RRMS patients identified according to their expression of CXCR3 and CCR6 (Figure 5D).

When we compared non-follicular Th subpopulations, decrease in the percentage of Th1 cells in 12 months treated RRMS patients was observed once compared to the untreated group (15.3 vs. 25.3%; $p < 0.01$) (Figure 5E). Moreover, we found an increase in the percentages of Th2 cells in 6 months treated patients (49.8 vs. 35.8%; $p < 0.01$) and an even higher increase in

12 months treated patients (56.4 vs. 35.8%; $p < 0.001$) compared to the untreated group (Figure 5G). We also detected a decreased percentage of non-follicular Th17.1 cells in 6 months treated patients (8.5 vs. 14.9%; $p < 0.05$) and an even higher decrease in 12 months treated patients (6.3 vs. 14.9%; $p < 0.001$) compared to untreated RRMS patients (Figure 5H). No differences were found when the effect of DMF on effector Th17 cells subpopulation was evaluated (Figure 5F).

When the effect of DMF on follicular cTfh effector subsets was separately evaluated, a lower percentage of cTfh1 cells in 12 months treated patients compared to untreated patients was found (17.4 vs. 25.5%; $p < 0.05$) (Figure 5I). The percentage of cTfh2 cells progressively increased significantly upon DMF treatment, being at 6 months (51.6 vs. 37.3%; $p < 0.01$) and at 12 months (55.1 vs.

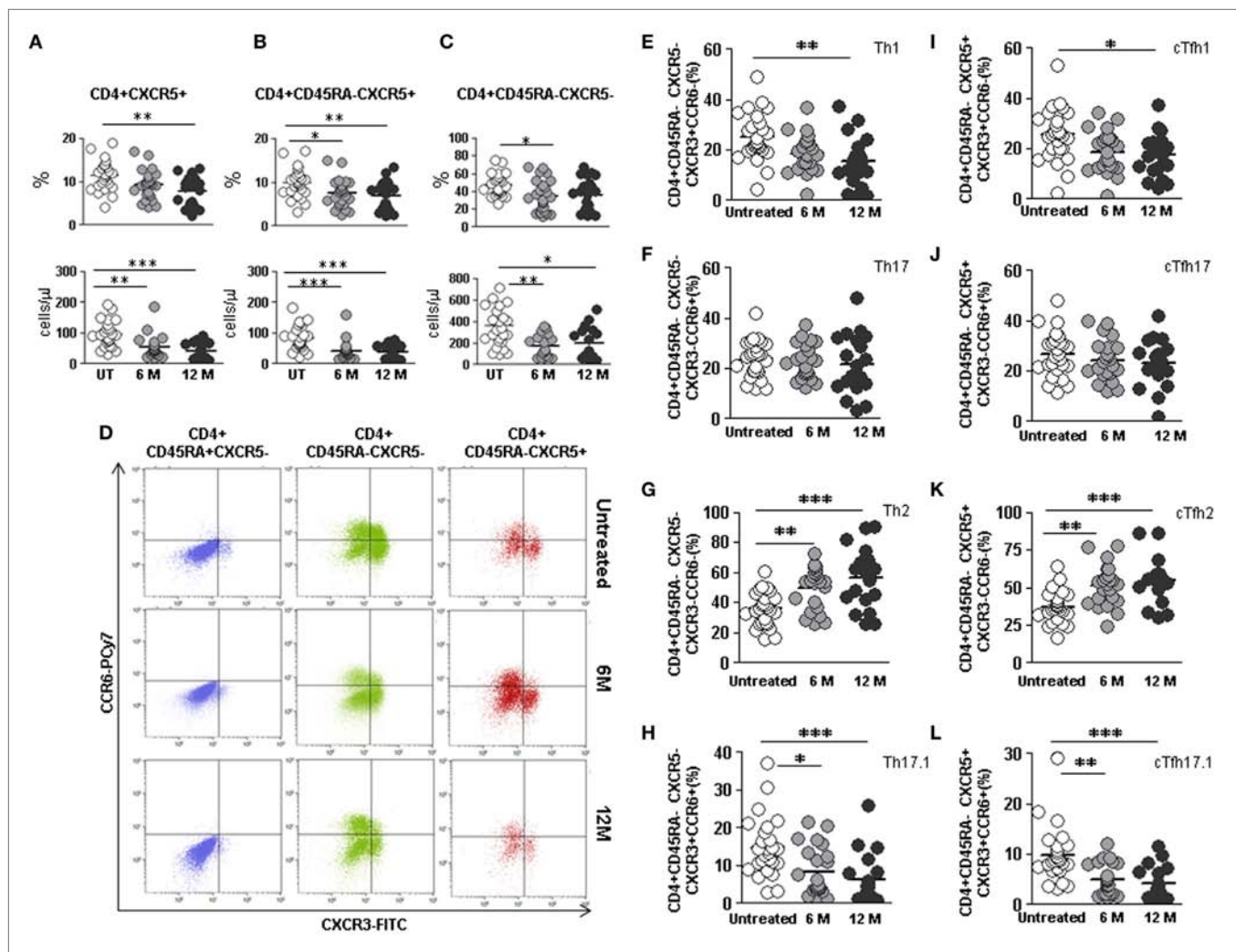


FIGURE 5 | Effect of dimethyl fumarate on total cTfh cells and subpopulations of Th and cTfh cells from multiple sclerosis (MS) patients. (A–C) Total follicular CD4+CXCR5+, memory follicular CD4+CD45RA–CXCR5+, and memory non-follicular CD4+CD45RA–CXCR5– percentages (upper panels) and cell counts (lower panels) from untreated (white circles), 6 months treated (6 M: light gray circles), and 12 months treated (12 M: dark gray circles) MS patients. (D) Dot-plots of the percentage of Th1 CXCR3+CCR6– (lower right quadrant), Th17 CXCR3–CCR6+ (upper left quadrant), Th2 CXCR3–CCR6– (lower left quadrant), and Th17.1 CXCR3+CCR6+ (upper right quadrant) subpopulations on circulating naïve CD4+CD45RA+CXCR5– (left), non-follicular CD4+CD45RA–CXCR5– (middle), and follicular CD4+CD45RA–CXCR5+ (right) cells from a representative untreated (upper row), 6 months treated (6 M: middle row), and 12 months treated (12 M: lower row) MS patient. (E–H) Percentages of Th1, Th17, Th2, and Th17.1 non-follicular T cells and (I–L) cTfh1, cTfh17, cTfh2, and cTfh17.1 follicular T cells from untreated (white circles), 6 months treated (6 M: light gray circles), and 12 months treated (12 M: dark gray circles) MS patients. Kruskal–Wallis test p -values: * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

37.3%; $p < 0.001$) (Figure 5K). Since we had observed an increase in the CXCR3+CCR6+ follicular cTfh17.1 population in RRMS untreated patients compared to controls (Figure 2L), we tested whether DMF treatment could modulate this subpopulation. Interestingly, we found a decrease in the percentage of cTfh17.1 in 6 months treated patients compared to untreated patients (5.1 vs. 9.9%; $p < 0.01$) that was even higher after 12 months treatment (4.2 vs. 9.9%; $p < 0.001$) (Figure 5L). Again, no differences were found when cTfh17 subpopulations were compared (Figure 5J).

Remarkably, we identified three non-responders patients in our cohort in whom DMF treatment had to be discontinued (patients 6, 8, and 18). When lymphocyte subpopulations of these particular patients were separately revised, we observed that DMF treatment had modified non-follicular Th subsets, but had not normalized the basal deviation of cTfh subpopulations: patient 6 had 37% cTfh2 and 9% cTfh17.1, patient 8 had 41% cTfh2 and 9% cTfh17.1 (both evaluated at 6 months), and patient 18 had 32% cTfh2 and 11% cTfh17.1 (evaluated at 12 months). In all cases, cTfh2 remained below percentile 25 (45%) and cTfh17.1 remained over percentile 75 (5%) of healthy subjects.

Moreover, patients 6 and 8 also maintained altered their B cells subpopulations upon 6 months DMF treatment. Patients 6 and 8 had 51 and 42% naïve B cells, respectively, both below percentile 25 of healthy subjects (52%). In addition, their switched memory B cells remained over percentile 75 of healthy subjects (19%), being 31 and 33%, respectively. Furthermore, these two patients (6 and 8) were the most affected at clinical evaluation on admission: EDSS > 3, more than 20 supratentorial lesions, ≥ 5

infratentorial lesions, and ≥ 2 spinal cord lesions in the MRI. Our data suggest that DMF may not be indicated for patients that are severely affected at disease onset.

Longitudinal Study Confirms Cross-Sectional Data

Longitudinal data derived from 14 RRMS patients, who could be followed both before and after 6 and 12 months DMF treatment, were analyzed to validate our cross-sectional findings. Overall, both Th cells and cTfh cells percentages decreased with time upon DMF treatment, confirming cross-sectional results (data not shown).

Specifically, percentages of Th1, Th17.1, cTfh1, and cTfh17.1 cells subpopulations progressively decreased whereas Th2 and cTfh2 progressively increased along DMF treatment (Figures 6A–H). Percentages of Th17 and cTfh17 cells were not significantly altered during the longitudinal study (Figures 6B,F), confirming the data obtained in the cross-sectional study (Figures 5E–L).

DMF Reverts the Pro-Inflammatory Shift of cTfh and B Cells to Healthy Subject's Values

To evaluate the final effect of DMF treatment, we compared the percentages of non-follicular Th, follicular cTfh effector, and B cells subpopulations on the RRMS group of patients at the end

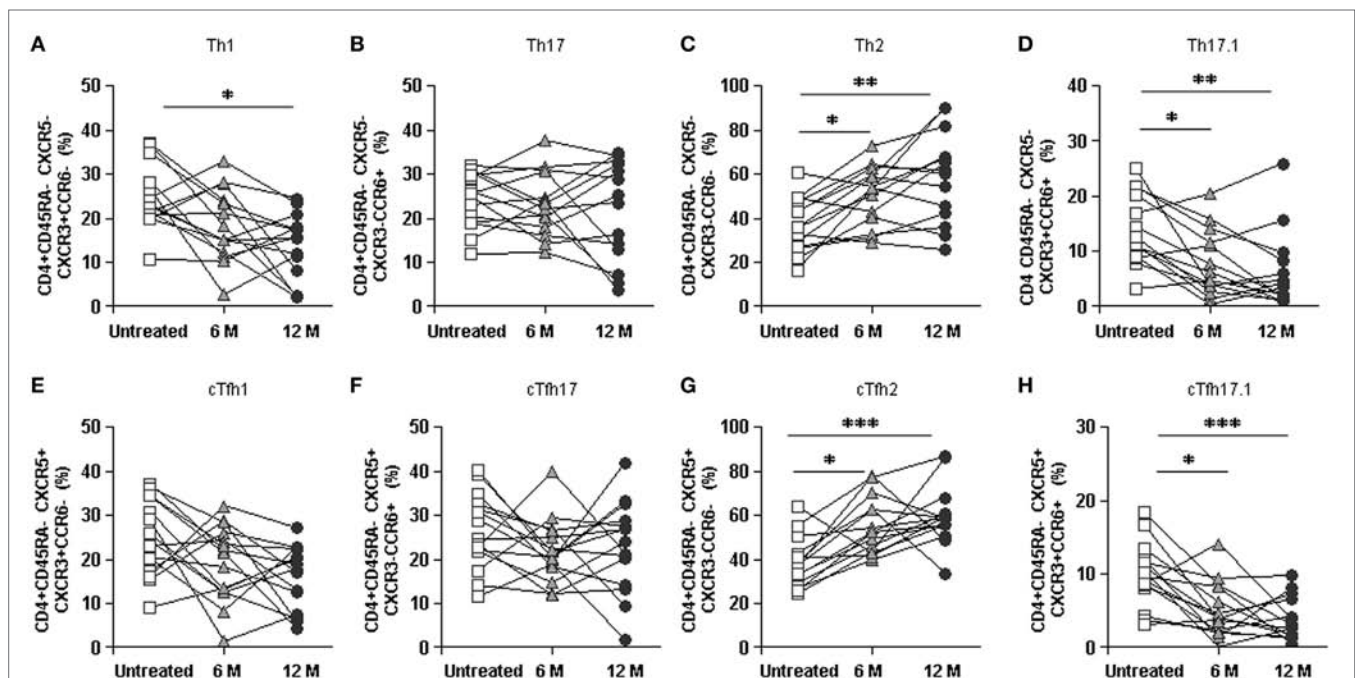
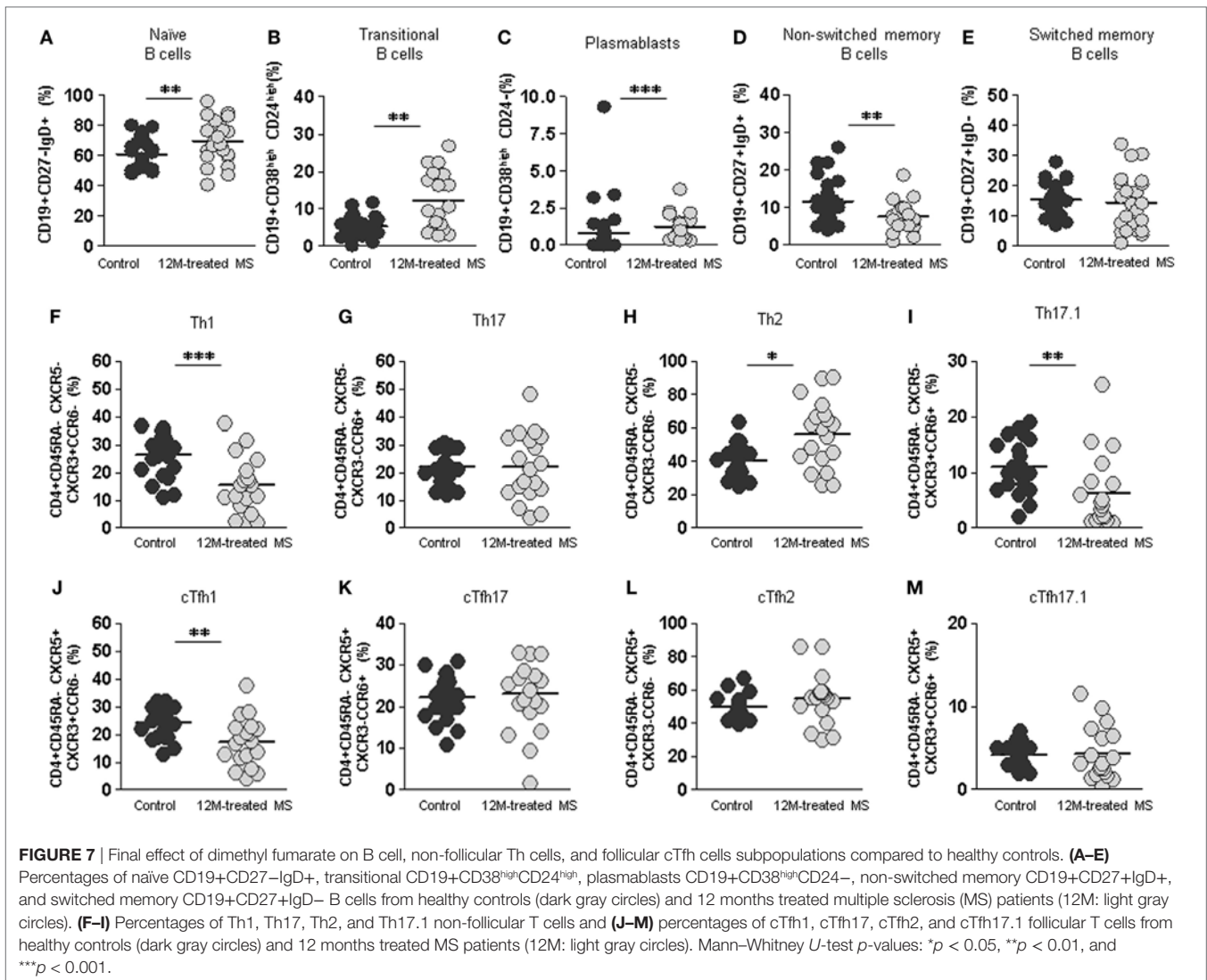


FIGURE 6 | Longitudinal study of the effect of dimethyl fumarate on Th and cTfh subpopulations from multiple sclerosis (MS) patients. (A–D) Percentages of Th1, Th17, Th2, and Th17.1 non-follicular T cells and (E–H) cTfh1, cTfh17, cTfh2, and cTfh17.1 follicular T cells from MS patients, before treatment (white squares) and 6 months (6 M: light gray triangles) and 12 months (12 M: dark gray circles) after treatment. Successive repeated measures on the same individual are represented by connecting lines. Friedman paired test p -values: * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.



of the 12 month treatment period with those of normal healthy subjects.

Remarkably, at this point of treatment, initially altered percentages of switched memory (CD19+CD27+IgD–) B cells returned to values of healthy subjects (**Figure 7E**). This was accompanied by a decrease of non-switched memory (CD19+CD27+IgD+) B cells to values significantly lower than those of healthy subjects (7.6 vs. 11.6%; $p < 0.01$) (**Figure 7D**). On the contrary, we found a significant increase in naïve (69.5 vs. 60.7%; $p < 0.01$), transitional (12.3 vs. 5.3%; $p < 0.01$), and plasmablasts (0.8 vs. 1.2%; $p < 0.001$) B cells at the end of the studied treatment period (**Figures 7A–C**).

After 12 month DMF treatment, percentages of both Th1 and Th17.1 non-follicular cells in 12 month treated RRMS group were even lower than those of healthy controls (15.3 vs. 26.4%; $p < 0.001$ and 6.3 vs. 11.1%; $p < 0.01$, respectively). Conversely, percentages of Th2 subpopulation were increased in the 12 month treated RRMS group compared to controls (56.4 vs. 40.3%; $p < 0.05$). This is consistent with an anti-inflammatory switch in non-follicular Th subpopulations induced by DMF treatment (**Figures 7F–I**).

When we evaluated effector follicular cTfh cells, cTfh1 were lower in the 12 month treated RRMS group compared to healthy controls (17.4 vs. 24.1%; $p < 0.01$); meanwhile, the percentages of cTfh2, cTfh17.1 did not differ between 12 month treated RRMS group and healthy controls (**Figures 7J–M**).

Thus, DMF reduces the absolute numbers of all major lymphocyte subpopulations, reverts the pro-inflammatory shift of the relevant cTfh and switched-memory B cells detected in untreated RRMS patients, and exerts a modifying effect in naïve, transitional, plasmablasts, and non-switched memory B cells subpopulations percentages.

DISCUSSION

Several immunological components have been implicated in the pathogenesis of MS with special relevance for CD4+ T cells (1), although an important role for B lymphocytes has also been demonstrated (6). We investigated the frequency and distribution of different lymphocyte subpopulations, with special focus on cTfh

cells, in RRMS patients compared to healthy subjects. Moreover, we evaluated whether these subpopulations could be modified in response to DMF treatment, and whether this potential shift could associate to treatment response in RRMS patients.

Although percentages and absolute counts of peripheral CD4+ and CD8+ T, NK, and B cells in our cohort of untreated RRMS patients were within reported ranges, distribution of B cells subsets was altered: the percentage of switched-memory B cells was increased.

cTfh cells have been previously found increased in MS patients (23) and ectopic lymphoid structures containing Tfh cells and B cells have been described in the meninges of MS patients, which could contribute to disease pathogenesis (5). Although we studied the subpopulations of non-follicular and Tfh cells, in our cohort of untreated RRMS patients, we only found important differences in the distribution of cTfh cells subpopulations. RRMS patients presented higher percentage of cTfh17.1 cells and lower percentage of cTfh2 cells, consistent with a pro-inflammatory bias only in cTfh subpopulations. cTfh17.1 cells express both CXCR3+CCR6+ and are analogous to the recently described Th17.1 helper effector subpopulation that produces high levels of IFN γ and IL-17 (16). Remarkably, Th17.1 subpopulation is resistant to glucocorticoids (16) and is increased in Crohn's disease (17) and in the lungs of sarcoidosis patients (16, 18).

Controversy exists about the implication of Th subpopulations and the role of IL-17 and IFN γ in the pathogenesis of MS. In RRMS patients, IL-17 levels were higher in serum and CSF (24) and IL-17-expressing CD4+ T cells were increased during relapses, while IFN γ -expressing CD4+ T cells remained stable (25). Moreover, myelin oligodendrocyte glycoprotein-specific CD4+ T cells in blood of RRMS patients were mostly CCR6 memory cells (5) producing higher levels of IFN γ , IL-17, and GM-CSF (26). Experiments in the experimental autoimmune encephalomyelitis MS mouse model have shown that Th17 cells induce ectopic lymphoid structures in the subarachnoid space, where they acquire a Tfh phenotype (27) and also that Th cells producing both IFN γ and IL-17 were more pathogenic than Th cells producing IFN γ or IL-17 alone (5). The fact that we found an increase in cTfh17.1 but not in cTfh17 may help to explain these controversies, as cTfh17.1 cells are able to produce both IFN γ and IL-17 cytokines. We suggest that the increase in cTfh17.1 and decrease in cTfh2 cells observed in our patients cohort may have a role in the pathogenesis of RRMS disease and could be a potential treatment target. Previous studies on other autoimmune diseases have also suggested that a skewed distribution of cTfh cell subsets contributes to their pathogenesis: higher levels of Tfh17 in primary Sjögren's syndrome (28) and also increased levels of both Tfh2 and Tfh17 in juvenile dermatomyositis (12) and Guillain-Barré syndrome (29).

Dimethyl fumarate is an approved oral treatment for RRMS. Recently, several articles addressing the effect of DMF in peripheral lymphocytes subpopulations have been published with the aim to underscore its mechanism of action and/or to find biological markers predicting treatment response. A reduction in lymphocyte counts and selective reductions in CD8+ T cells (30) or memory T cells (31) have been described after DMF treatment. An anti-inflammatory shift in B cells subsets has also

been described; with decrease of the memory B cells pool and reduction in GM-CSF, IL-6, and TNF α expressing B cells (32).

Our 29 patients cross-sectional study confirmed a decrease in the absolute numbers but not in the percentages of CD4+ and CD8+ T cells, B, and NK cells, consistent with the lymphopenia described in DMF-treated patients (9). However, an important shift was detected in B cells subpopulations distribution: while switched memory B cells significantly decreased, a significant increase of naïve, transitional, and plasmablasts B cells was observed, whereas non-switched memory B cells remained unaltered.

We observed that DMF induced a progressive decrease of non-follicular Th1 and an increase in the anti-inflammatory Th2 subpopulation, whereas Th17 subpopulation remained unchanged, suggesting an anti-inflammatory shift in previously unaltered Th subpopulations. Our results are consistent with previous reports in which a decrease in Th1, increase in Th2, and no alteration of Th17 memory T cells was reported in 15 RRMS patients treated only 6 months with DMF (33). A reduction in Th1 (analyzed as CXCR3+) and increase in Th2 (analyzed as CCR3+) frequencies were also reported in a nine patients longitudinal cohort (9). However, in contrast to our study, these authors also found a reduction in Th17 cells. Discrepancy in the results may be due to the experimental protocol used to analyze Th17 subpopulation: their analysis was based on "CCR6+ only," which presumably includes CCR6+CXCR3- non altered Th17 cells, but also our separately analyzed CCR6+CXCR3+ Th17.1 cells, the subpopulation which we have identified to be reduced by DMF treatment.

Our most relevant finding comes from the study of cTfh subpopulations, which are responsible for driving B cell differentiation. Important differences were found between untreated and DMF-treated patients: a significant increase of cTfh2 cells was observed in 12 month treated patients, accompanied by a significant decrease in cTfh1 and cTfh17.1 cells. No differences were found in cTfh17 percentages. These results imply that DMF reverts the pro-inflammatory switch of cTfh cells detected in untreated RRMS patients. B cells modifications found after treatment could be an indirect consequence of the observed cTfh subpopulations modulation, but we cannot rule out a direct effect of DMF on B cells.

In any case, DMF-induced changes in these subpopulations had a humoral consequence. Although serum levels of IgG and IgM were not importantly modified, IgA significantly decreased over time in response to DMF treatment. This is an important observation as a recent report has demonstrated a relationship between CSF IgA levels and IgA CSF/serum quotient, and cerebral atrophy and EDS in MS patients (34). Moreover, given the importance of several cytokines, particularly IFN γ , in the IgG subclass switch process (35, 36), changes in IgG subclass profile (decrease in IgG3 and IgG2) in the serum of our DMF-treated patients favor a role of the Tfh subpopulations shift and their secreted cytokines in the DMF treatment effect.

When we compared untreated RRMS patients with healthy subjects, we only found differences in the cTfh subpopulations (higher percentage of Tfh17.1 and lower percentage of Tfh2 cells) and B cells subpopulations (increase in switched memory B). Over the course of our study, DMF progressively reverted

this proinflammatory shift in cTfh and B cells subpopulations by the end of the 12-month treatment if compared to healthy controls values. Interestingly, three DMF non-responder patients did not revert the deviation of cTfh subpopulations. Although these findings should be validated with wider cohorts of DMF non-responders, our data suggest that normalization of cTfh subpopulations upon DMF treatment may be related to clinical response. Moreover, the fact that two of these patients were the most severely affected at baseline evaluation may suggest that DMF is not effective in those cases and treatment should be started earlier.

In summary, we have demonstrated a pro-inflammatory shift of cTfh and B cells in RRMS patients. DMF treatment induces a progressive decrease in cTfh1 and the pathogenic cTfh17.1 subpopulations, together with an increase in cTfh2 subpopulations, resulting in a reversion of this situation. This effect is accompanied by a corresponding decrease in switched-memory B cells and an increase in naïve, transitional, and plasmablasts B cells. We postulate that the decrease in pro-inflammatory IFN γ and IL-17 producing cells and antigen experienced B cells may explain the protective activity of DMF in RRMS-treated patients. The measurement of cTfh subpopulations could be a biological marker to evaluate DMF response.

ETHICS STATEMENT

The study was conducted according to the ethical guidelines of the 1975 Declaration of Helsinki and approved by CEIC (Balearic

Islands Clinical Research Ethics Committee). Written informed consent was obtained from all subjects.

AUTHOR CONTRIBUTIONS

VC performed the experiments and conducted the acquisition, interpretation, and statistical analysis of data. MM, CC, VN, and RD conducted patients recruitment and clinical information monitoring. AC collaborated with the analysis and interpretation of data and critical revision of the manuscript. VA, AL-G, and MJ collaborated with the acquisition and analysis of data. JP collaborated with the critical revision of the manuscript. CV-B designed research project and collaborated with the critical revision of the manuscript. JF designed research project, generated funding, and wrote the manuscript.

ACKNOWLEDGMENTS

The excellent technical assistance of Cristina Martínez, Marina Montes, Dimas García, and Bernat Ortega-Vila is gratefully acknowledged. We thank all patients in the cohort that donated their blood to perform this study.

FUNDING

This work has been supported by the Fondo de Investigación Sanitaria (grant number: FIS PI14/00265) from the Instituto de Salud Carlos III (Spanish Government) and the Fondo Europeo de Desarrollo Regional (FEDER).

REFERENCES

- Dendrou CA, Fugger L, Friese MA. Immunopathology of multiple sclerosis. *Nat Rev Immunol* (2015) 15:545–58. doi:10.1038/nri3871
- Antel J, Antel S, Caramanos Z, Arnold DL, Kuhlmann T. Primary progressive multiple sclerosis: part of the MS disease spectrum or separate disease entity? *Acta Neuropathol* (2012) 123:627–38. doi:10.1007/s00401-012-0953-0
- Hoglund RA, Maghazachi AA. Multiple sclerosis and the role of immune cells. *World J Exp Med* (2014) 4:27–37. doi:10.5493/wjem.v4.i3.27
- Dargahi N, Katsara M, Tselios T, Androutsou ME, de Courten M, Matsoukas J, et al. Multiple sclerosis: Immunopathology and treatment update. *Brain Sci* (2017) 7(7):78. doi:10.3390/brainsci7070078
- Schmitt N. Role of T follicular helper cells in multiple sclerosis. *J Nat Sci* (2015) 1(7):e139.
- Gelfand JM, Cree BAC, Hauser SL. Ocrelizumab and other CD20+ B-cell-depleting therapies in multiple sclerosis. *Neurotherapeutics* (2017) 14(4):835–41. doi:10.1007/s13311-017-0557-4
- Vargas DL, Tyor WR. Update on disease-modifying therapies for multiple sclerosis. *J Investig Med* (2017) 65:883–91. doi:10.1136/jim-2016-000339
- Linker RA, Lee DH, Ryan S, van Dam AM, Conrad R, Bista P, et al. Fumaric acid esters exert neuroprotective effects in neuroinflammation via activation of the Nrf2 antioxidant pathway. *Brain* (2011) 134:678–92. doi:10.1093/brain/awq386
- Wu Q, Wang Q, Mao G, Dowling CA, Lundy SK, Mao-Draayer Y. Dimethyl fumarate selectively reduces memory T cells and shifts the balance between Th1/Th17 and Th2 in multiple sclerosis patients. *J Immunol* (2017) 198:3069–80. doi:10.4049/jimmunol.1601532
- Breitfeld D, Ohl L, Kremmer E, Ellwart J, Sallusto F, Lipp M, et al. Follicular B helper T cells express CXC chemokine receptor 5, localize to B cell follicles, and support immunoglobulin production. *J Exp Med* (2000) 192:1545–52. doi:10.1084/jem.192.11.1545
- Schaerli P, Willmann K, Lang AB, Lipp M, Loetscher P, Moser B. CXC chemokine receptor 5 expression defines follicular homing T cells with B cell helper function. *J Exp Med* (2000) 192:1553–62. doi:10.1084/jem.192.11.1553
- Morita R, Schmitt N, Bentebibel SE, Ranganathan R, Bourdery L, Zurawski G, et al. Human blood CXCR5(+)CD4(+) T cells are counterparts of T follicular cells and contain specific subsets that differentially support antibody secretion. *Immunity* (2011) 34:108–21. doi:10.1016/j.immuni.2011.01.009
- Fan X, Lin C, Han J, Jiang X, Zhu J, Jin T. Follicular helper CD4+ T cells in human neuroautoimmune diseases and their animal models. *Mediators Inflamm* (2015) 2015:638968. doi:10.1155/2015/638968
- Qiu H, Wu H, Chan V, Lau CS, Lu Q. Transcriptional and epigenetic regulation of follicular T-helper cells and their role in autoimmunity. *Autoimmunity* (2017) 50:71–81. doi:10.1080/08916934.2017.1284821
- Ma CS, Wong N, Rao G, Avery DT, Torpy J, Hambridge T, et al. Monogenic mutations differentially affect the quantity and quality of T follicular helper cells in patients with human primary immunodeficiencies. *J Allergy Clin Immunol* (2015) 136:993–1006.e1. doi:10.1016/j.jaci.2015.05.036
- Ramesh R, Kozhaya L, McKevitt K, Djuretic IM, Carlson TJ, Quintero MA, et al. Pro-inflammatory human Th17 cells selectively express P-glycoprotein and are refractory to glucocorticoids. *J Exp Med* (2014) 211:89–104. doi:10.1084/jem.20130301
- Ramstein J, Broos CE, Simpson LJ, Ansel KM, Sun SA, Ho ME, et al. IFN- γ -producing T-helper 17.1 cells are increased in sarcoidosis and are more prevalent than T-helper type 1 cells. *Am J Respir Crit Care Med* (2016) 193:1281–91. doi:10.1164/rccm.201507-1499OC
- Tondell A, Moen T, Borset M, Salvesen O, Ro AD, Sue-Chu M. Bronchoalveolar lavage fluid IFN- γ + Th17 cells and regulatory T cells in pulmonary sarcoidosis. *Mediators Inflamm* (2014) 2014:438070. doi:10.1155/2014/438070
- Cunill V, Clemente A, Lanio N, Barcelo C, Andreu V, Pons J, et al. Follicular T cells from smB- common variable immunodeficiency patients are

- skewed toward a Th1 phenotype. *Front Immunol* (2017) 8:174. doi:10.3389/fimmu.2017.00174
20. Polman CH, Reingold SC, Banwell B, Clanet M, Cohen JA, Filippi M, et al. Diagnostic criteria for multiple sclerosis: 2010 revisions to the McDonald criteria. *Ann Neurol* (2011) 69:292–302. doi:10.1002/ana.22366
 21. Wong WS, Lo AW, Siu LP, Leung JN, Tu SP, Tai SW, et al. Reference ranges for lymphocyte subsets among healthy Hong Kong chinese adults by single-platform flow cytometry. *Clin Vaccine Immunol* (2013) 20:602–6. doi:10.1128/CVI.00476-12
 22. Boldt A, Borte S, Fricke S, Kentouche K, Emmrich F, Borte M, et al. Eight-color immunophenotyping of T-, B-, and NK-cell subpopulations for characterization of chronic immunodeficiencies. *Cytometry B Clin Cytom* (2014) 86:191–206. doi:10.1002/cytob.21162
 23. Fan X, Jin T, Zhao S, Liu C, Han J, Jiang X, et al. Circulating CCR7+ICOS+ memory T follicular helper cells in patients with multiple sclerosis. *PLoS One* (2015) 10(7):e0134523. doi:10.1371/journal.pone.0134523
 24. Tao Y, Zhang X, Chopra M, Kim MJ, Buch KR, Kong D, et al. The role of endogenous IFN-beta in the regulation of Th17 responses in patients with relapsing-remitting multiple sclerosis. *J Immunol* (2014) 192:5610–7. doi:10.4049/jimmunol.1302580
 25. Brucklacher-Waldert V, Stüerner K, Kolster M, Wolthausen J, Tolosa E. Phenotypical and functional characterization of T helper 17 cells in multiple sclerosis. *Brain* (2009) 132:3329–41. doi:10.1093/brain/awp289
 26. Cao Y, Goods BA, Raddassi K, Nepom GT, Kwok WW, Love JC, et al. Functional inflammatory profiles distinguish myelin-reactive T cells from patients with multiple sclerosis. *Sci Transl Med* (2015) 7:287ra74. doi:10.1126/scitranslmed.aaa8038
 27. Peters A, Pitcher LA, Sullivan JM, Mitsdoerffer M, Acton SE, Franz B, et al. Th17 cells induce ectopic lymphoid follicles in central nervous system tissue inflammation. *Immunity* (2011) 35:986–96. doi:10.1016/j.immuni.2011.10.015
 28. Li XY, Wu ZB, Ding J, Zheng ZH, Li XY, Chen LN, et al. Role of the frequency of blood CD4(+) CXCR5(+) CCR6(+) T cells in autoimmunity in patients with Sjogren's syndrome. *Biochem Biophys Res Commun* (2012) 422:238–44. doi:10.1016/j.bbrc.2012.04.133
 29. Che Y, Qiu J, Jin T, Yin F, Li M, Jiang Y. Circulating memory T follicular helper subsets, Tfh2 and Tfh17, participate in the pathogenesis of Guillain-Barre syndrome. *Sci Rep* (2016) 6:20963. doi:10.1038/srep20963
 30. Spencer CM, Crabtree-Hartman EC, Lehmann-Horn K, Cree BA, Zamvil SS. Reduction of CD8(+) T lymphocytes in multiple sclerosis patients treated with dimethyl fumarate. *Neurol Neuroimmunol Neuroinflamm* (2015) 2:e76. doi:10.1212/NXI.0000000000000076
 31. Longbrake EE, Ramsbottom MJ, Cantoni C, Ghezzi L, Cross AH, Piccio L. Dimethyl fumarate selectively reduces memory T cells in multiple sclerosis patients. *Mult Scler* (2016) 22:1061–70. doi:10.1177/1352458515608961
 32. Li R, Rezk A, Ghadiri M, Luessi F, Zipp F, Li H, et al. Dimethyl fumarate treatment mediates an anti-inflammatory shift in B cell subsets of patients with multiple sclerosis. *J Immunol* (2017) 198:691–8. doi:10.4049/jimmunol.1601649
 33. Gross CC, Schulte-Mecklenbeck A, Klinsing S, Posevitz-Fejfar A, Wiendl H, Klotz L. Dimethyl fumarate treatment alters circulating T helper cell subsets in multiple sclerosis. *Neurol Neuroimmunol Neuroinflamm* (2015) 3:e183. doi:10.1212/NXI.0000000000000183
 34. Kroth J, Ciolac D, Fleischer V, Koirala N, Krämer J, Muthuraman M, et al. Increased cerebrospinal fluid albumin and immunoglobulin A fractions forecast cortical atrophy and longitudinal functional deterioration in relapsing-remitting multiple sclerosis. *Mult Scler* (2017) 1:1352458517748474. doi:10.1177/1352458517748474
 35. Inoue R, Kondo N, Kobayashi Y, Fukutomi O, Orii T. IgG2 deficiency associated with defects in production of interferon-gamma; comparison with common variable immunodeficiency. *Scand J Immunol* (1995) 41:130–4. doi:10.1111/j.1365-3083.1995.tb03544.x
 36. Kawano Y, Noma T, Yata J. Regulation of human IgG subclass production by cytokines. IFN-gamma and IL-6 act antagonistically in the induction of human IgG1 but additively in the induction of IgG2. *J Immunol* (1994) 153:4948–58.

Conflict of Interest Statement: VC, AC, VA, AL-G, RD, MJ, JP, CV-B, and JF declare no conflict of interest. MM, CC, and VN have been speakers on behalf of Biogen.

Copyright © 2018 Cunill, Massot, Clemente, Calles, Andreu, Núñez, López-Gómez, Díaz, Jiménez, Pons, Vives-Bauzá and Ferrer. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



Correlates of Follicular Helper Bias in the CD4 T Cell Response to a Retroviral Antigen

Luca Danelli¹, Tiziano Donnarumma¹ and George Kassiotis^{1,2*}

¹ Retroviral Immunology, The Francis Crick Institute, London, United Kingdom, ² Department of Medicine, Faculty of Medicine, Imperial College London, London, United Kingdom

OPEN ACCESS

Edited by:

Georgia Fousteri,
San Raffaele Hospital (IRCCS), Italy

Reviewed by:

Hu Zeng,
Mayo Clinic, United States
Thomas Ciucci,
National Cancer Institute (NCI),
United States

*Correspondence:

George Kassiotis
george.kassiotis@crick.ac.uk

Specialty section:

This article was submitted
to T Cell Biology,
a section of the journal
Frontiers in Immunology

Received: 28 March 2018

Accepted: 18 May 2018

Published: 05 June 2018

Citation:

Danelli L, Donnarumma T and
Kassiotis G (2018) Correlates
of Follicular Helper Bias in
the CD4 T Cell Response
to a Retroviral Antigen.
Front. Immunol. 9:1260.
doi: 10.3389/fimmu.2018.01260

CD4⁺ T cell differentiation is influenced by a plethora of intrinsic and extrinsic factors, providing the immune system with the ability to tailor its response according to specific stimuli. Indeed, different classes of pathogens may induce a distinct balance of CD4⁺ T cell differentiation programmes. Here, we report an uncommonly strong bias toward follicular helper (Tfh) differentiation of CD4⁺ T cells reactive with a retroviral envelope glycoprotein model antigen, presented in its natural context during retroviral infection. Conversely, the response to the same antigen, presented in different immunization regimens, elicited a response typically balanced between Tfh and T helper 1 cells. Comprehensive quantitation of variables known to influence Tfh differentiation revealed the closest correlation with the strength of T cell receptor (TCR) signaling, leading to PD-1 expression, but not with surface TCR downregulation, irrespective of TCR clonotypic avidity. In contrast, strong TCR signaling leading to TCR downregulation and induction of LAG3 expression in high TCR avidity clonotypes restrained CD4⁺ T cell commitment and further differentiation. Finally, stunted Th1 differentiation, correlating with limited IL-2 availability in retroviral infection, provided permissive conditions for Tfh development, suggesting that Tfh differentiation is the default program of envelope-reactive CD4⁺ T cells.

Keywords: CD4 T cell response, follicular helper T cells, retroviral infection, CD4 T cell differentiation, TH1 T cells, vaccine vectors and adjuvants

INTRODUCTION

Several divergent and often competing programmes of CD4⁺ T cell differentiation are now well recognized, leading to the development of distinct functional subsets, including T helper (Th) 1, 2, or 17 cells, follicular helper (Tfh) cells, and regulatory T (Treg) cells (1–5). The relative balance of CD4⁺ T cell differentiation to one or more of these functional subsets largely depends on a multitude of T cell-extrinsic factors, with the cytokine milieu naïve T cells encounter during the priming phase playing a major role (1–5). However, CD4⁺ T cell differentiation can also be shaped by T cell-intrinsic properties, such as the relative affinity of the T cell receptor (TCR), favoring development of particular subsets (6–8). The combined effect of such T cell-extrinsic and T cell-intrinsic factors can result in considerable diversity of functional responses, allowing adaptive immunity to modify its response according to the nature of the antigenic stimulus.

Acute viral infections typically induce a CD4⁺ T cell response that is almost exclusively composed of Tfh and Th1 cells, in approximately equal proportion. Indeed, the ratio of Tfh to Th1 cells in the CD4⁺ T cell response to acute lymphocytic choriomeningitis virus (LCMV) has been amply reported

close to 1 and 2 for LCMV Armstrong (9–14) and clone 13 (Cl13) (15–19), respectively, and similar results were also reported for influenza A virus infection (20–23).

Several well-defined factors have been demonstrated to influence the balance of Th1 and Tfh cells in response to viruses, as well as other challenges. One of these is the availability of IL-2, determined both by the rate of production by effector CD4⁺ T cells and the rate of consumption by Treg cells or dendritic cells (20, 24–26). IL-2 has been reported to negatively affect Tfh differentiation, in favor of Th1 differentiation (20, 24–26). The balance of Th1 and Tfh differentiations in response to infection with viruses, as well as other classes of pathogens, is also strongly influenced by the nature of the dominant antigen-presenting cell (APC) type (2, 3, 5, 6). For example, antigen presentation by B cells is considered critical for consolidating Tfh differentiation, whereas presentation by macrophages is thought to promote Th1 differentiation (2, 3, 5, 6).

How T cells integrate the multitude of intrinsic and extrinsic factors regulating the balance of their differentiation is not currently completely understood. It is possible that these factors do not operate independently, but are linked at the level of APC-T cell interaction. Indeed, the type of dominant APC determines the cytokine milieu (e.g., IL-12 production or IL-2 consumption) (20, 24–26), the provision of costimulatory signals (e.g., ICOS-L) (5, 27), and the TCR signal strength, given that APC types differ in the potency of stimulation.

We studied the CD4⁺ T cell response to model retroviral antigen from the gp70 envelope glycoprotein of the Friend murine leukemia virus (F-MLV) (28, 29), to assess the relative contribution of individual intrinsic and extrinsic factors, previously linked to the balance of Th1 and Tfh differentiations. Here, we report an atypically strong bias toward Tfh differentiation in response to this retroviral antigen, when presented during natural infection, and describe the variables of the immune response that determine the balance of Th1 and Tfh differentiations in this setting.

MATERIALS AND METHODS

Mice

Inbred C57BL/6 (B6) and CD45.1⁺ congenic B6 (B6.SJL-*Ptprca* *Pep3b*/BoyJ) mice were originally obtained from The Jackson Laboratory (Bar Harbor, ME, USA). TCRβ-transgenic EF4.1 mice (30), Nur77-GFP transgenic mice (31), B cell receptor-deficient (*Ighm*^{−/−}) (32), Tcrα-deficient (*Tcrα*^{−/−}) (33), Rag1-deficient (*Rag1*^{−/−}) mice (34), and Rag2-deficient (*Rag2*^{−/−}) mice (35) were on the B6 genetic background and were maintained at the Francis Crick Institute's animal facilities. All animal experiments were approved by the ethical committee of the Francis Crick Institute and conducted according to local guidelines and UK Home Office regulations under the Animals Scientific Procedures Act 1986 (ASPA).

CD4⁺ T Cell Adoptive Transfer

Single-cell suspensions were prepared from the spleens of donor CD45.1⁺ or CD45.2⁺ TCRβ-transgenic EF4.1 mice or CD45.2⁺ Nur77-GFP EF4.1 doubly transgenic mice, and CD4⁺ T cells were enriched using an immunomagnetic positive selection

kit (StemCell Technologies, Vancouver, BC, Canada), at >90% purity. Donor transgenic CD4⁺ T cells (1 × 10⁶ per recipient) were injected into recipient mice intravenously.

Retroviral Infection and Immunization

The Friend virus (FV) used in this study was a retroviral complex of a replication-competent B-tropic F-MLV (F-MLV-B) and a replication-defective spleen focus-forming virus (SFFV). Stocks were prepared as previously described (36). Mice were injected intravenously with 0.1 mL PBS containing ~50 (low dose), 1,000 (intermediate dose), or 5,000 (high dose) spleen focus-forming units of FV. The F-MLV-NB env_{L128I} variant was generated by mutagenesis of the respective codon (CTC → ATT) in plasmid pLRB302, containing the complete NB-tropic F-MLV clone FB29. The resulting plasmid was then transfected into *Mus dunni* fibroblast cells (*M. dunni* cells; CRL-2017). Stocks of F-MLV-B, F-MLV-NB env_{L128I}, or F-MLV-N helper virus were grown in *M. dunni* cells. Mice received an inoculum of ~10⁴ infectious units of helper virus by intravenous injection. Ad5.pIX-gp70 stocks were prepared at a titer of 9 × 10⁹ viral genomes per milliliter by infection of 293A cells as previously described (37). Approximately 5 × 10⁸ Ad5.pIX-gp70 viral genomes per mouse were administered intravenously. Immunization with FBL-3 tumor cells was carried out by intravenous injection of 1.5 × 10⁶ FBL-3 cells (38). For peptide immunization, mice received an intraperitoneal injection of a total of 12.5 nmol of synthetic env₁₂₂₋₁₄₁ peptide mixed in Sigma Adjuvant System (Sigma-Aldrich, St. Louis, MO, USA). Where indicated, recipient mice also received blocking antibodies against PD-1 (10 mg/kg, clone RMP1-14) and LAG3 (10 mg/kg, clone C9B7W) (both from BioXCell, West Lebanon, NH, USA), injected intraperitoneally on days 0, 1, 3, and 5 post FV infection.

Antibodies and Flow Cytometry

Spleen single-cell suspensions were stained for 20 min at room temperature or at 4°C with directly conjugated antibodies to surface markers. For detection of intracellular antigens, subsequent to surface staining, cells were fixed and permeabilized using the Foxp3/Transcription Factor Staining Buffer Set (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. They were then incubated for 45 min at room temperature with directly conjugated antibodies to intracellular antigens. Zombie UV Fixable Viability Kit (BioLegend, San Diego, CA, USA) was used to label and exclude dead cells from analysis. The following anti-mouse antibodies were used: BV785- or BV711-anti-CD4 (clone GK1.5), PE/Cy7-anti-CD45.1 (clone A20), PE/Cy7-anti-CD279 (PD-1, clone 29F.1A12), BV785-anti-CD150 (SLAMF7, clone TC15-12F12.2) (from BioLegend); V500-anti-CD44 (clone IM7), BV421- or PerCP-Cy5.5-anti-CD162 (PSGL1, clone 2PH1), BV421-anti-Ly6C (clone AL-21), PE-anti-Bcl6 (clone K112-91), FITC-anti-Vα2 (clone B20.1) (from BD Biosciences, San Jose, CA, USA); PE-anti-CD25 (clone PC61.5), PE-Cyanine7-anti-CD45R (B220, clone RA3-6B2), APC-eFluor-780-anti-CD45.2 (clone 104), eFluor450-anti-CD45.1 (clone A20), PE-anti-CD223 (LAG3, clone eBioC9B7W), APC-anti-Ter119 (clone TER-119), APC-anti-Vα2 (clone B20.1), FITC- or APC-anti-TCRβ (clone H57-597) (from Thermo Fisher Scientific, Waltham, MA, USA); Alexa(R)488- or Alexa(R)647-anti-TCF1 (clone C63D9) (from

Cell Signaling Technology, Danvers, MA, USA). For CXCR5 staining, splenocytes were incubated with biotin rat anti-mouse CXCR5 antibody (clone 2G8, BD Biosciences) at 37°C for 25 min, followed by incubation with APC- or PE-streptavidin (BioLegend) for 20 min at room temperature. FV-infected cells were detected by using surface staining for the glycosylated product of the viral gag gene (Glyco-Gag), using the matrix (MA)-specific monoclonal antibody 34 (mouse IgG2b), followed by an FITC-anti-mouse IgG2b secondary reagent (clone 12-3 from BD). Multi-color cytometry was performed on LSRFortessa flow cytometers (from BD Biosciences) and analyzed with FlowJo v10.1 (Tree Star Inc., Ashland, OR, USA).

Fluorescence Microscopy

Frozen OCT (Dako)-embedded spleen sections were fixed in cold acetone, stained with fluorescein labeled peanut agglutinin (PNA, Vector Laboratories), and with directly conjugated antibodies against anti-mouse/human B220 (clone RA3-6B2, AlexaFluor 594, BioLegend) and anti-mouse CD45.1 (clone A20, Alexa Fluor 647, BioLegend). Stained sections were mounted in fluorescent mounting medium (Dako) and viewed with an Olympus IX83 inverted microscope system (Olympus Corporation, Shinjuku, Tokyo, Japan).

Analysis of Single-Cell RNA-Sequencing Data

Gene transcription in env-reactive CD4⁺ T cells was assessed using publicly available single-cell RNA-sequencing data (European Nucleotide Archive accession number PRJEB14043) as previously described (39). These included the transcriptional profiles of single env-reactive donor CD4⁺ T cells isolated from the spleens of wild-type (WT) recipients infected with FV or immunized with Ad5.pIX-gp70, 7 days previously. They also included the transcriptional profiles of single env-reactive donor EF4.1 CD4⁺ T cells that carried a WT *Bcl6* allele (*Bcl6*^{wt}) or a conditional *Bcl6* allele (*Bcl6*^{fl}), purified from the spleens of WT recipient mice, 7 days after FV infection. Expression values were analyzed using the Qlucore Omics Explorer 3.3 (Qlucore, Lund, Sweden), and pathway analyses were performed using The Database for Annotation, Visualization and Integrated Discovery v6.8 (<https://david.ncifcrf.gov/home.jsp>).

Cytokine Gene Transcription and Protein Production

Serum levels of IL-2 were measured on a Luminex system (BioPlex 100) using the mouse cytokine kits (Bioplex Mouse cytokine group II and Bioplex Mouse Cytokine Standard; Bio-Rad Laboratories, Hercules, CA, USA) following the manufacturer's instructions, as previously described (30, 40). Transcription of the indicted cytokine genes in env-reactive CD4⁺ T cells was assessed using publicly available single-cell RNA-sequencing data, as described above.

Statistical Analyses

Statistical comparisons were made using SigmaPlot 13.0 (Systat Software Inc., Germany) or GraphPad Prism 7 (GraphPad

Software, La Jolla, CA, USA). Parametric comparisons of normally distributed values that satisfied the variance criteria were made by unpaired Student's *t*-tests or One Way Analysis of variance (ANOVA) tests. Data that did not pass the variance test were compared with non-parametric two-tailed Mann-Whitney Rank Sum tests or ANOVA on Ranks tests. *p* Values are indicated by asterisks as follows: **p* < 0.05; ***p* < 0.005; ****p* < 0.0005. Hierarchical clustering, principal component analysis, and heat-map production were performed with Qlucore Omics Explorer 3.3 (Qlucore).

RESULTS

The CD4⁺ T Cell Response to F-MLV Env Is Heavily Dominated by Tfh Cells

To study CD4⁺ effector T cell development, we employed a well-described adoptive transfer system, where EF4.1 TCRβ-transgenic CD4⁺ T cells, reactive with the dominant H2-A^b-restricted env₁₂₂₋₁₄₁ epitope within the F-MLV gp70 glycoprotein, were transferred into WT B6 recipients (37, 41). Transferred T cells were primed in recipient mice by infection with FV, a retroviral complex of F-MLV and SFFV that causes chronic infection in B6 mice (28, 29).

As previously reported (41, 42), a considerable proportion (~50%) of env-reactive EF4.1 CD4⁺ T cells developed a PD-1^{high} CXCR5⁺ phenotype consistent with Tfh cells (Figure 1A). Further detailed phenotypic characterization confirmed the Tfh profile of these cells as CXCR5⁺ PD-1^{high} Bcl6⁺ PSGL1⁻ Ly6C⁻ SLAMF6^{low} (Figure 1B). Consistent with their phenotype, a large proportion of donor CD4⁺ T cells localized within B cell follicles or germinal centers in the spleens of recipient mice (Figure 1C), further supporting strong Tfh differentiation of env-reactive EF4.1 CD4⁺ T cells.

Notable, however, were the relative paucity (~10%) of env-reactive EF4.1 CD4⁺ T cells with a Th1 phenotype (PSGL1⁺ PD-1⁺ SLAMF6⁺ CXCR5⁻ Bcl6⁻ Ly6C⁻) (43) and the presence of a sizable population (~35%) of cells that lacked markers of Tfh or Th1 commitment (PSGL1⁻ SLAMF6⁻ CXCR5⁻) (Figures 1A,B). The latter population, referred here as Th0 to denote their uncommitted state (44), retained TCF-1 expression and expressed low levels of Bcl6 (Figure 1B).

As antigen availability can greatly influence CD4⁺ T cell differentiation, we compared the efficiency of Tfh development in response to different FV loads (Figure 1D). Surprisingly, the proportion of seemingly uncommitted Th0 cells correlated inversely with levels of FV infection, both in terms of absolute numbers and proportion within env-reactive donor CD4⁺ T cells (Figures 1B,E). Indeed, whereas the highest dose of FV primed increased absolute numbers of all three EF4.1 CD4⁺ T cell subsets, Th0 cells exhibited the highest increase (Figure 1E). In contrast, the lowest dose of FV elicited considerable reduced numbers of Th0 and Th1 cells, while favoring development of Tfh cells, which now comprised the overwhelming majority (Figure 1E).

Thus, FV infection induces primarily a Tfh phenotype in env-reactive EF4.1 CD4⁺ T cells and, to a much lesser extent, a Th1 phenotype (Figure 1E), whereas Th2, Th17, Treg, or CTL

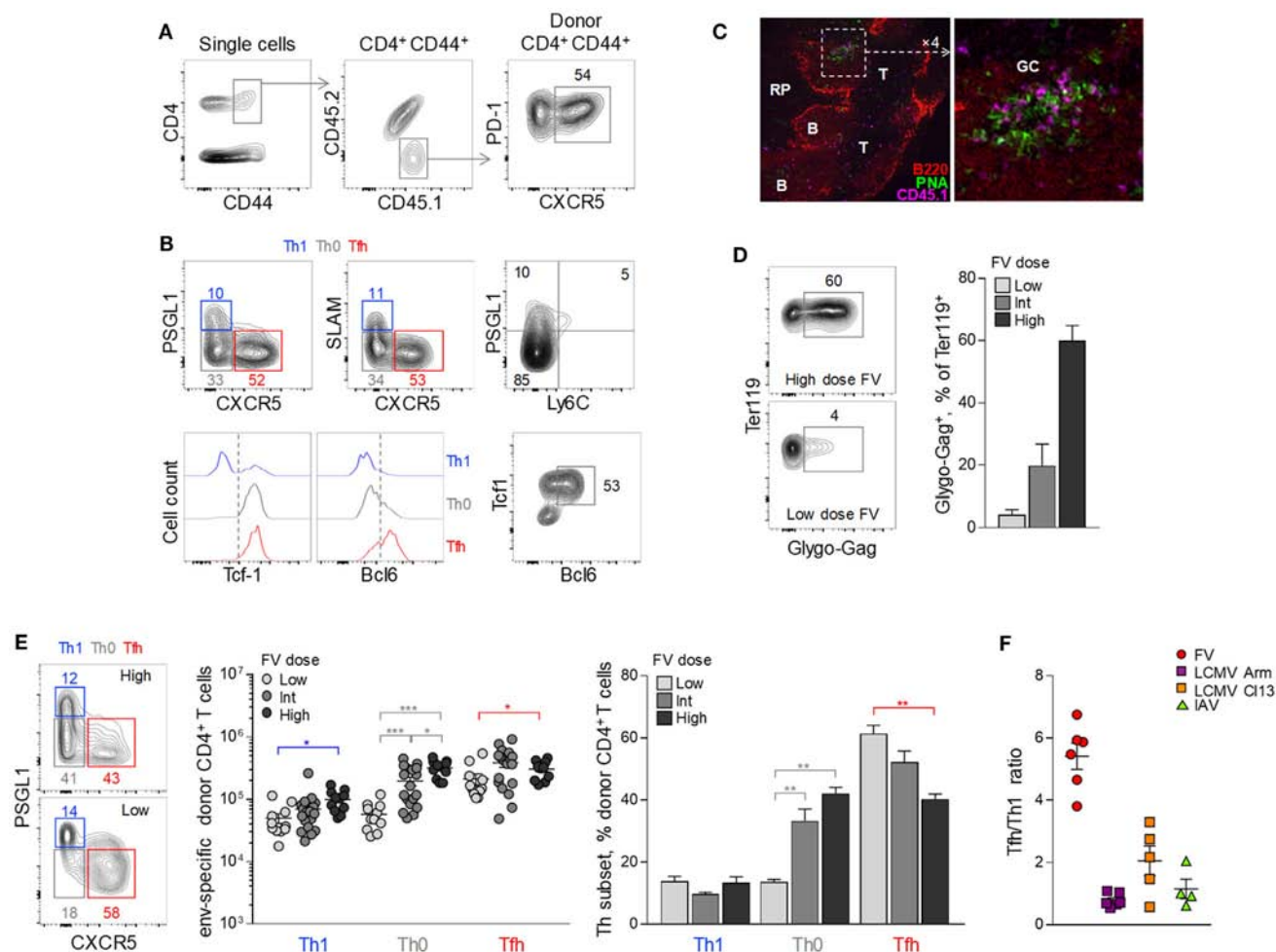


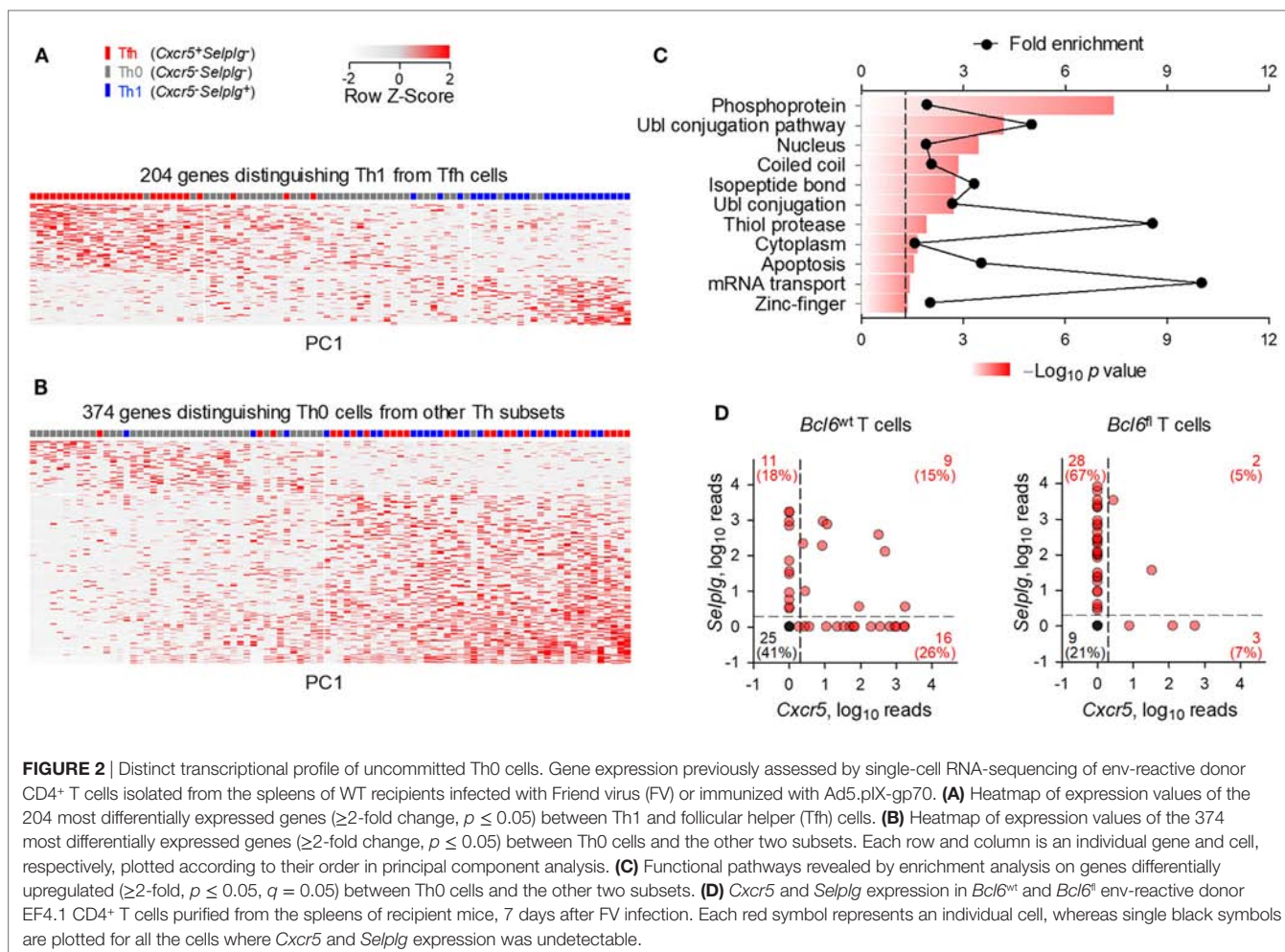
FIGURE 1 | Follicular helper (Tfh) cells heavily dominate the CD4⁺ T cell response to F-MLV env. **(A,B)** CD4⁺ TCRβ-transgenic EF4.1 T cells were adoptively transferred into CD45.1⁺ CD45.2⁺ WT recipients infected with an intermediate dose of Friend virus (FV) (1,000 spleen focus-forming units) and analyzed by flow cytometry in the spleen 7 days later. **(A)** Gating strategy for the identification of env-reactive (CD4⁺ CD44⁺) and Tfh-phenotype (PD-1⁺ CXCR5⁺) donor cells in these recipients. **(B)** Flow cytometric characterization of Th1 (in blue), Th0 (in gray), and Tfh (in red) subsets in env-reactive donor CD4⁺ CD44⁺ T cells. **(C)** CD45.1⁺ CD4⁺ EF4.1 T cells were adoptively transferred into FV-infected CD45.2⁺ WT recipients and detected by immunofluorescence microscopy in spleen sections 7 days later (RP, red pulp; B, B cell zone; T, T cell zone; GC, germinal center). Donor CD4⁺ were identified as CD45.1⁺ cells and germinal centers were visualized by staining with peanut agglutinin (PNA). **(D)** Flow cytometric example of FV-infected Glyco-Gag⁺ Ter119⁺ cells (left) and frequency of Glyco-Gag⁺ cells in the Ter119⁺ population (right) in the spleens of WT B6 mice infected with different doses of FV. Mean frequency (±SEM, *n* = 3 mice per group) of one representative of two experiments is shown. **(E)** Profile of PSGL1 and CXCR5 expression (left), absolute number (middle), and mean frequency (±SEM) (right) of Th1, Th0, and Tfh cells in env-reactive donor CD4⁺ T cells from the spleen of recipient mice infected with the indicated doses of FV (data are from 4–6 experiments for each FV dose with at least three mice per experiment). **(F)** Tfh/Th1 ratio in env-reactive donor CD4⁺ T cells after FV infection (individual symbols denote the mean Tfh/Th1 ratio in independent experiments with at least 3 mice per experiment), compared the Tfh/Th1 ratio reported in the literature for virus-specific CD4⁺ T cells responding to infection with lymphocytic choriomeningitis virus (LCMV) Armstrong (Arm), LCMV clone 13 (Cl13) or influenza virus PR38 (IAV).

differentiation is not typically observed (37, 39, 42). This skewing in favor of Tfh differentiation was much more pronounced in FV infection, where there are on average 5.2 times more Tfh than Th1 cells, than in other acute viral infections, where this ratio is consistently reported to be closer to 1 (9–23) (Figure 1F).

Distinguishable Transcriptional Activity of Uncommitted Th0 Cells

The strong bias in Tfh development following FV infection suggested that the seemingly uncommitted Th0 cells were primed

env-reactive CD4⁺ T cell that had not successfully completed the program of Tfh differentiation. To better place them in the spectrum of Th differentiation, we compared the transcriptional profiles of Th0 cells using single-cell RNA-sequencing data obtained with env-specific EF4.1 CD4⁺ T cells primed by FV or a replication-defective human adenovirus serotype 5 vector expressing the F-MLV gp70 glycoprotein (Ad5.pIX-gp70) (39). Th1, Tfh, and Th0 cells were defined here according to their expression of *Cxcr5* and *Selplg* (the gene encoding PSGL1). We first selected the top 204 genes, whose expression best differentiated Th1 and Tfh cells (≥2-fold change, *p* ≤ 0.05) (Figure 2A).



Transcription of these genes in Th0 cells showed a profile that was intermediate between the Th1 and Tfh extremes (**Figure 2A**), consistent with their unpolarized phenotype. Direct comparison between Th0 cells and the other two subsets revealed an extensive set of genes that were differentially expressed (≥ 2 -fold change, $p \leq 0.05$), the majority of which were absent from Th0 cells (**Figure 2B**; Table S1 in Supplementary Material). Specific to Th0 cells was expression of 88 genes (Table S1 in Supplementary Material), involved in active metabolic pathways (**Figure 2C**), indicating a highly activated phenotype of Th0 cells, despite incomplete differentiation.

To further probe any subset commitment of Th0 cells, albeit incomplete, we examined their dependency on *Bcl6* expression. This was achieved by using single-cell RNA-sequencing data obtained with env-specific EF4.1 CD4⁺ T cells that carried a WT *Bcl6* allele (*Bcl6*^{wt}) or a conditional *Bcl6* allele (*Bcl6*^{fl}) that was deleted by Cre-mediated recombination upon T cell activation (39). When primed by FV in WT hosts, 41% (25/61) of *Bcl6*^{wt} EF4.1 CD4⁺ T cells displayed a Th0 phenotype (*Cxcr5*⁺*Selplg*⁺), whereas this proportion was reduced to 21% (9/42) in *Bcl6*^{fl} EF4.1 CD4⁺ T cells (**Figure 2D**), despite comparable numerical priming of both types of T cell under these conditions (39). Together, these data suggested that at least a proportion of Th0 cells may have

initiated but not completed Tfh differentiation, particularly in conditions of high viral loads.

Inhibitory Receptors Restrain Full Tfh Maturation in Response to FV Infection

To further probe how FV loads might influence Tfh differentiation of env-reactive EF4.1 CD4⁺ T cells, we examined the potential effect of inhibitory receptors. At the peak of their response to FV infection, env-reactive EF4.1 CD4⁺ T cells have been previously shown to express high levels of multiple inhibitory receptors, including PD-1 and LAG3 (39). Analysis of the earliest time-points at which donor CD4⁺ T cell expansion can be reliably demonstrated (**Figure 3A**) revealed that PD-1 and LAG3 expression reached near maximum levels already by day 4 post FV infection (**Figure 3B**).

Also comparable between days 4 and 7, post infection was the relative ratio of Tfh and Th1 cells in env-reactive EF4.1 CD4⁺ T cells (**Figure 3C**), in agreement with commitment to Tfh or Th1 differentiation at this early time-point (45). Given that proliferation of donor CD4⁺ T cells is atypically slowing down between days 4 and 7 post FV infection (37), we reasoned that the early induction of inhibitory receptors during FV infection might restrict further differentiation. Consistent with this notion,

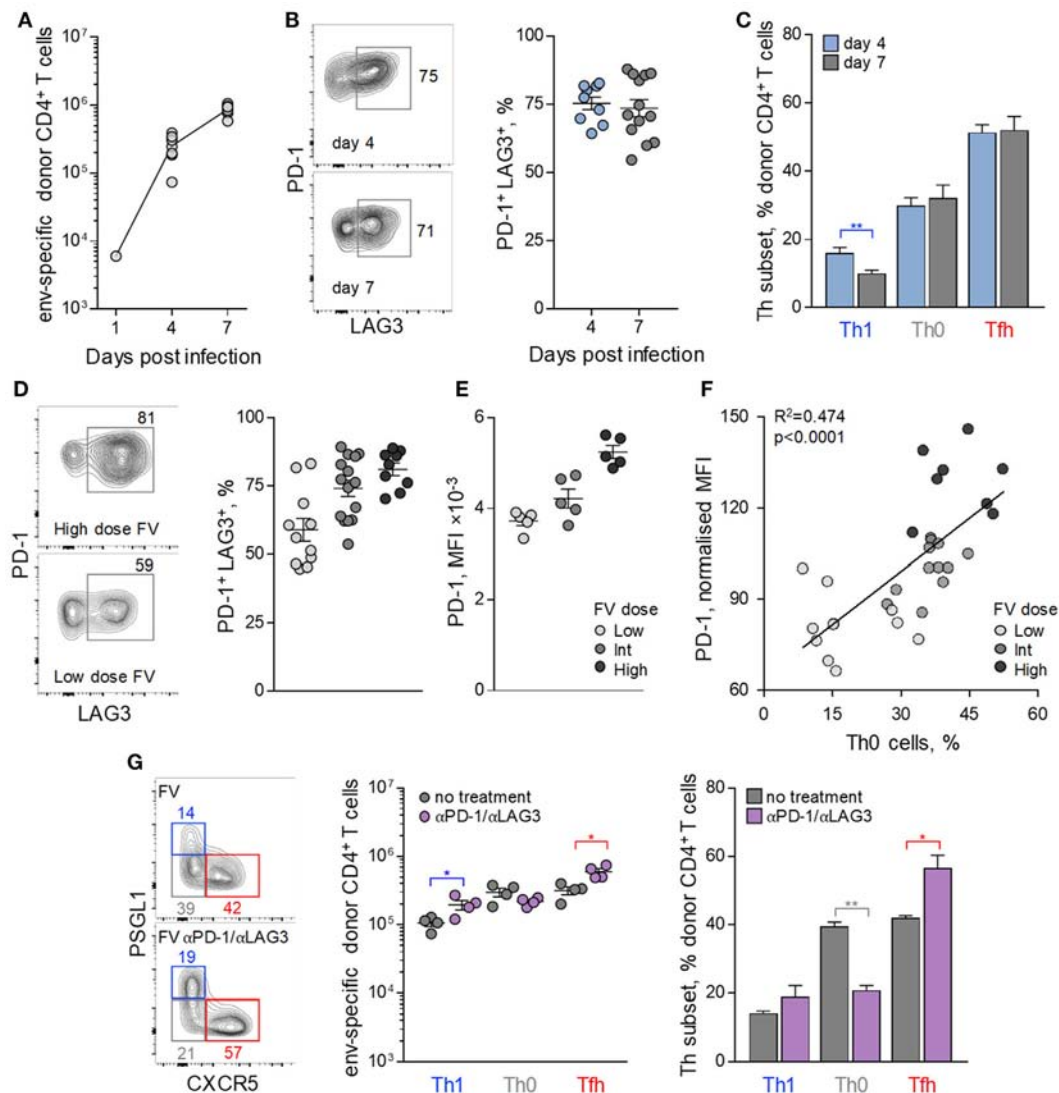


FIGURE 3 | Follicular helper (Tfh) cells maturation control by inhibitory receptor induction. CD4⁺ EF4.1 T cells were adoptively transferred into WT recipients infected with intermediate dose of Friend virus (FV) and analyzed by flow cytometry in the spleens on days 4 ($n = 9$) and 7 ($n = 13$) post-transfer and infection. **(A)** Absolute number of env-reactive CD4⁺ T cells were recovered. **(B)** Flow cytometry profile (left) and mean frequency (±SEM) (right) of PD-1⁺ LAG3⁺ cells within env-reactive donor CD4⁺ T cells. Each symbol is an individual mouse. **(C)** Mean frequency (±SEM) of T helper (Th) subsets, defined by PSGL1 and CXCR5 expression, in env-reactive donor CD4⁺ T cells. **(D)** Representative flow cytometric profile of PD-1 and LAG3 expression (left), mean frequency (±SEM) of PD-1⁺ LAG3⁺ cells, and **(E)** mean fluorescence intensity (MFI) of PD-1 staining in env-reactive CD4⁺ T cells isolated from the spleens of recipient mice on day 7 post transfer and infection with the indicated dose of FV. Each symbol is an individual mouse ($n = 11$, $n = 14$, and $n = 9$ for low, intermediate and high FV dose, respectively). **(F)** Correlation between the frequency of Th0 cells and the MFI of PD-1 expression in env-reactive donor CD4⁺ T cells from the same mice described in (D). **(G)** Flow cytometric analysis (left), absolute number (middle), and mean frequency (±SEM) (right) of Th subsets, defined by PSGL1 and CXCR5 expression, in env-reactive donor CD4⁺ T cells, 7 days after transfer into FV-infected recipients with or without additional treatment with PD-1 and LAG3 blocking antibodies ($n = 4$ from one of two representative experiments).

frequencies of env-reactive EF4.1 CD4⁺ T cells coexpressing PD-1 and LAG3, as well as the intensity of PD-1 expression, directly correlated with both FV loads and the proportion of the uncommitted Th0 subset of env-reactive EF4.1 CD4⁺ T cells (Figures 3D–F).

To test whether the early expression of PD-1 and LAG3 during FV infection was impeding Th differentiation of env-reactive EF4.1 CD4⁺ T cells, we treated recipient mice with PD-1 and

LAG3 blocking antibodies. Such treatment during FV infection was previously demonstrated to promote CTL differentiation of donor CD4⁺ T cells, which is not typically observed in FV infection (39). However, the CD4⁺ CTL subset induced by PD-1 and LAG3 blockade during FV infection constituted only ~7% of env-reactive EF4.1 CD4⁺ T cells (39), and it was possible that Tfh differentiation was still favoured. Indeed, PD-1 and LAG3 blockade during FV infection drove efficient differentiation of

uncommitted Th0 env-reactive EF4.1 CD4⁺ T cells into Tfh cells, which again formed the large majority, with a small numerical increase in Th1 cells and a small numerical loss of Th0 cells (Figure 3G).

Together, these results suggested that FV infection induces primarily Tfh differentiation of env-reactive EF4.1 CD4⁺ T cells, partly restrained by inhibitory receptor expression, in turn induced by strong TCR signaling.

Multifactorial Contribution to Tfh Cell Development During FV Infection

The ability of FV infection to promote Tfh differentiation, especially under conditions when PD-1 and LAG3 were not maximally expressed or were blocked, seemed rather exceptional. A host of well-established intrinsic and extrinsic factors may help or hinder Tfh development and, alone or in combination, could account for the dominance of Tfh cells in the response to FV infection. Included among these factors are the avidity of the TCR, the form of antigen and duration of its presentation, the strength of interaction with B cells, and the cytokine environment, particularly the availability of IL-2.

We next investigated how modulation of one or more variables known to affect Tfh cell development, might shape differentiation of CD4⁺ T cells in response to FV. High TCR avidity has long been suggested as a contributor to Tfh bias also in response to FV infection (6–8). EF4.1 CD4⁺ T cells comprise a semi-polyclonal repertoire of env-reactive TCRs, differing in their avidity for the cognate antigen, according to the pairing of the transgenic TCR β chain with endogenous TCR α chains (30, 41). Indeed, clonotypes with higher or lower TCR avidity for H2-A^b-env₁₂₂₋₁₄₁ can be identified by the use of TCR V α 2 or V α 3 (non-V α 2) endogenous chains, respectively, and used to examine the effect of TCR avidity on Th differentiation (30, 41). Nevertheless, Tfh development was broadly comparable between V α 2⁺ and V α 3⁺ env-reactive CD4⁺ T cell clonotypes, albeit development of Th1 cells appeared more efficient in lower avidity V α 3⁺ clonotypes in response to FV infection (Figure 4A). Thus, the bias in Tfh differentiation of EF4.1 CD4⁺ T cells was observed across clonotypes with a range of TCR avidities.

As an independent way to assess the effect of TCR avidity, we introduced mutations that alter the potency of the F-MLV env₁₂₂₋₁₄₁ epitope to stimulate particular clonotypes. Prior work highlighted the L residue at position 128 as an important contributor to recognition, particularly by higher avidity V α 2⁺ env-reactive CD4⁺ T cell clonotypes (46). Epitopes carrying an L128I mutation behave as strong agonists for all clonotypes (46). F-MLVs expressing variants of the env₁₂₂₋₁₄₁ epitope were also compared with N-tropic F-MLV (F-MLV-N), whose replication in B6 mice is restricted by the product of the *FvI^b* allele, therefore reducing the amount of available antigen.

Friend murine leukemia virus carrying the env_{L128I} epitope induced significantly higher numbers of env-reactive EF4.1 CD4⁺ T cells than WT F-MLV-B, whereas F-MLV-N was less immunogenic (Figure 4B). Consistent with its potency, F-MLV-NB env_{L128I} infection primed V α 2⁺ as well as V α 3⁺

env-reactive CD4⁺ T cell clonotypes, in contrast to WT F-MLV infection, which favored V α 2⁺ clonotypes (Figure 4B). As a result, the frequency of V α 2⁺ clonotypes in env-reactive CD4⁺ T cells was higher following infection with WT F-MLV than with F-MLV-NB env_{L128I} (Figure 4B). Moreover, expression of inhibitory receptors PD-1 and LAG3 was also regulated according to the potency of antigenic stimulation, reaching maximal levels in response to F-MLV env_{L128I} infection (Figure 4B). However, despite notable differences in induced clonal expansion and potency of antigenic stimulation between the mutant F-MLV viruses, differentiation of primed EF4.1 CD4⁺ T cells was highly comparable and still heavily skewed toward the Tfh subset (Figure 4C).

Th differentiation in response to a given antigen is also influenced by the context in which it is presented and in particular by its vector. We, therefore, compared the degree of Tfh differentiation of EF4.1 CD4⁺ T cells induced by F-MLV env₁₂₂₋₁₄₁ in different immunization regimens. These included vaccination with Ad5.pIX-gp70 (47), transient env₁₂₄₋₁₃₈ peptide immunization in Sigma adjuvant and transplantation of the FV-induced FBL-3 tumor cell line (38).

Each immunization regimen induced a characteristic pattern of clonotypic composition and inhibitory receptor expression (Figure 5A). In comparison with FV infection, Ad5.pIX-gp70 immunization induced a sizeable Tfh, as well as Th1 population of env-reactive CD4⁺ T cells, as previously shown for CD4⁺ CTL development (39), without concomitant increases in the Th0 subset (Figures 5A,B). Peptide immunization induced lower numbers of env-reactive CD4⁺ T cells, but this reduction affected mostly Th0 and Tfh cells (Figures 5A,B). Finally, FBL-3 tumor cell immunization promoted expansion of Th1 cells, at the expense of Tfh cells, but also primed Th0 cells (Figures 5A,B). Thus, each immunization regimen elicited a somewhat distinct Th differentiation balance in env-reactive EF4.1 CD4⁺ T cells. Nevertheless, Tfh cells continued to be a prominent subset in response to all the regimens (Figure 5B).

Finally, the Tfh differentiation was examined in the context of altered lymphocyte interaction. To this end, EF4.1 CD4⁺ T cells were transferred into FV-infected hosts deficient in B cells (*Ighm*^{−/−}), T cells (*Tcra*^{−/−}), or both B and T cells (*Rag2*^{−/−}). Again, clonotypic composition or inhibitory receptor expression in the env-reactive donor CD4⁺ T cells was characteristic of each type of recipient (Figure 6A). Consistent with the established role for B cells in consolidating Tfh differentiation, env-reactive EF4.1 CD4⁺ T cells produced a larger fraction of Th1 cells in *Ighm*^{−/−} hosts, than in WT hosts (Figures 6A,B). Expectedly, enhanced Th1 differentiation in B cell-deficient hosts was at the expense of Tfh differentiation (Figures 6A,B). Despite this shift, however, Th1-phenotype env-reactive EF4.1 CD4⁺ T cells in *Ighm*^{−/−} hosts did not outnumber those with a Tfh phenotype or those with an uncommitted Th0 phenotype, with all three subsets represented in almost equal proportions (Figure 6B). Surprisingly, in comparison with WT or *Ighm*^{−/−} hosts, Th1 differentiation of env-reactive EF4.1 CD4⁺ T cells was significantly more pronounced in *Tcra*^{−/−} hosts, where Th1 cells now became the dominant subset (Figures 6A,B). Skewed differentiation was even more pronounced in *Rag2*^{−/−} hosts, where

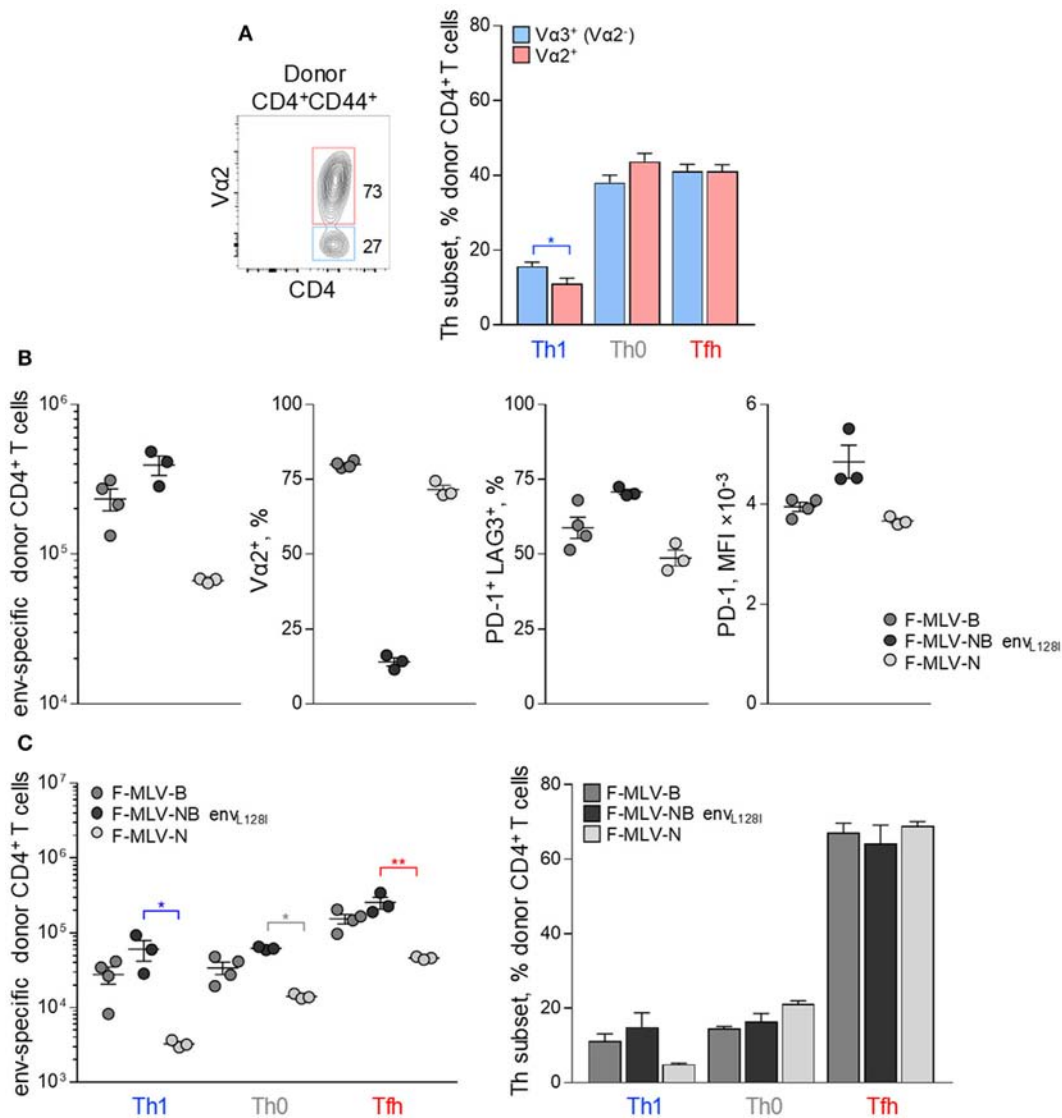


FIGURE 4 | Effect of TCR avidity on the follicular helper cells (Tfh) response to F-MLV env. **(A)** Flow cytometry example of Va2 expression (left) and mean frequency ($\pm$ SEM) of T helper (Th) subsets, defined by PSGL1 and CXCR5 expression, in Va2⁺ or Va3⁺ (Va2⁻) env-reactive donor CD4⁺ T cells, 7 days after transfer into Friend virus (FV) infected recipients ($n = 10$). **(B)** Characterization of clonal expansion and expression of Va2, PD-1, and LAG3 in env-reactive donor CD4⁺ T cells, 7 days post-transfer in WT recipient mice infected with $\sim 10^4$ infectious units of F-MLV-B, F-MLV-NB env_{L128I}, or F-MLV-N. **(C)** Absolute number (left) and mean frequency ($\pm$ SEM) (right) of Th subsets, defined by PSGL1 and CXCR5 expression, in env-reactive donor CD4⁺ T cells from the same donor cells as in **(B)**. One representative of two experiments with $n = 4$, $n = 3$, and $n = 3$ mice for F-MLV-B, F-MLV-NB env_{L128I}, and F-MLV-N infection, respectively, is shown.

env-reactive EF4.1 CD4⁺ T cells developed almost exclusively ($\sim 75\%$) into Th1 cells (Figures 6A,B). This shift in favor of Th1 differentiation in *Tcra*^{-/-} and *Rag2*^{-/-} hosts was driven by approximately 60-fold higher expansion of Th1 cells in such T cell-lymphopenic hosts, in comparison with T cell-replete hosts.

Together, these results highlighted the multitude of intrinsic and extrinsic factors that influence the balance of Tfh and Th1 differentiation in response to F-MLV env, but also indicated that each of these factors may contribute to a different degree, with T cell lymphopenia exerting the strongest influence.

Reduced IL-2 Availability During FV Infection Facilitates Tfh Development

In addition to increased availability of antigenic peptide-MHC complexes, a well-described effect of T cell lymphopenia is reduced T cell competition for other growth signals, such as cytokines. Consumption of effector CD4⁺ T cell-produced IL-2 by Treg cells or dendritic cells has been shown to promote Tfh differentiation, at the expense of Th1 differentiation in a variety of experimental systems (20, 24–26). It was, therefore, possible that the strong bias toward Tfh differentiation in FV infection was due to defective IL-2 signaling, either due to lack of production

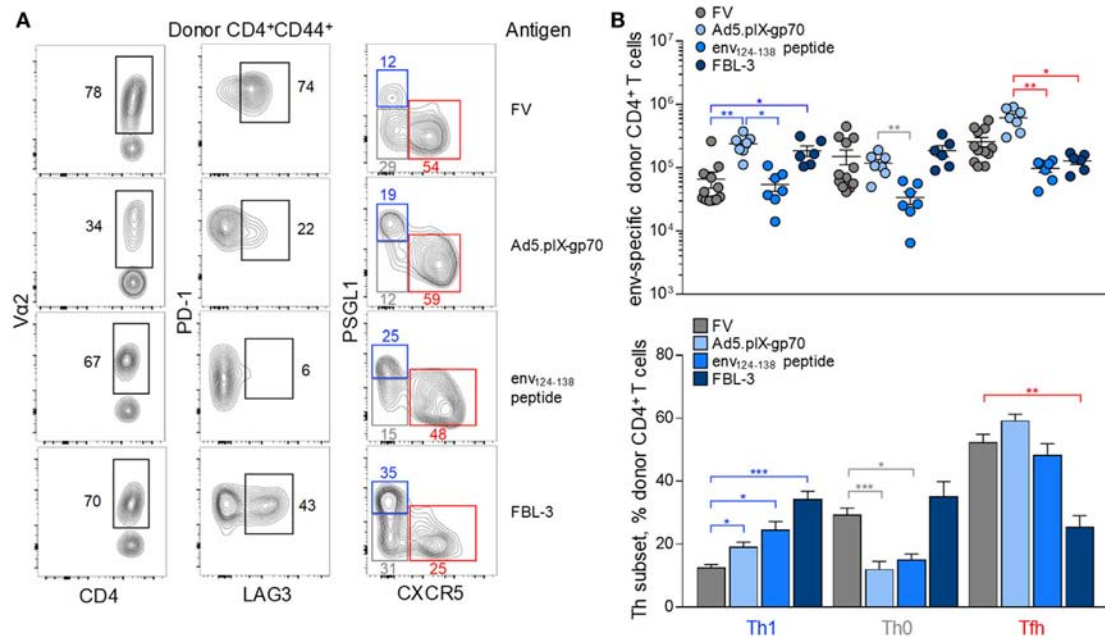


FIGURE 5 | Effect of immunization regimen on the follicular helper cells (Tfh) response to F-MLV env. **(A)** Flow cytometric analysis of Vα2, PD-1, LAG3, PSGL1, and CXCR5 expression in env-reactive CD4⁺ T cells isolated from the spleens of WT recipients on day 7 post transfer and priming with the indicated immunization regimens [intermediate Friend virus (FV) infection, $n = 3$; Ad5.pIX-gp70 vaccination, $n = 7$; env₁₂₄₋₁₃₈ peptide in Sigma Adjuvant System immunization, $n = 7$; FBL-3 challenge, $n = 6$]. **(B)** Absolute number (top) and mean frequency ($\pm$ SEM) (bottom) of T helper (Th) subsets, defined by PSGL1 and CXCR5 expression, in env-reactive donor CD4⁺ T cells from the same donor cells as in **(A)**.

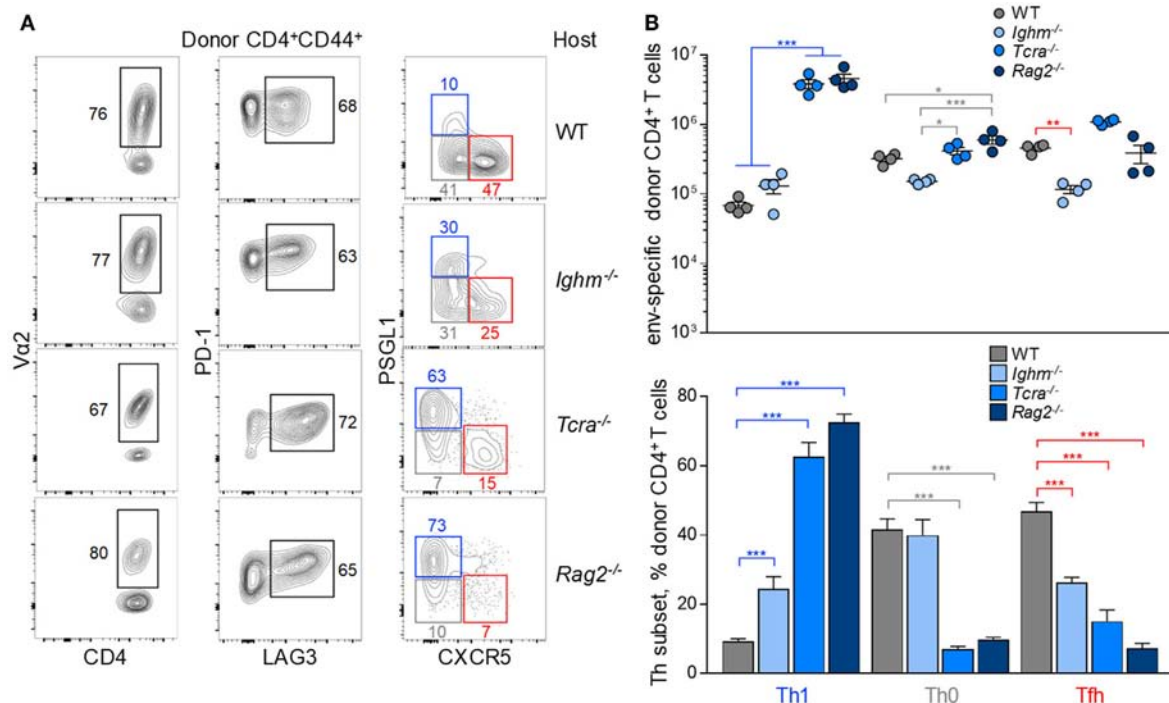


FIGURE 6 | Effect of lymphocyte interaction on the follicular helper (Tfh) response to F-MLV env. **(A)** Flow cytometric analysis of Vα2, PD-1, LAG3, PSGL1, and CXCR5 expression in env-reactive CD4⁺ T cells isolated from the spleens of the indicated recipient mice on day 7 post transfer and intermediate Friend virus (FV) infection (WT host, $n = 12$; Ighm^{-/-} host, $n = 6$; Tcra^{-/-} host, $n = 6$; Rag2^{-/-} host, $n = 7$). **(B)** Absolute number (top) and mean frequency ($\pm$ SEM) (bottom) of T helper (Th) subsets, defined by PSGL1 and CXCR5 expression, in env-reactive donor CD4⁺ T cells from the same donor cells as in **(A)**.

or to increased consumption by cells other than effector CD4⁺ T cells.

Consistent with this hypothesis, expression levels of CD25 were lower when env-specific EF4.1 CD4⁺ T cells were primed in WT than in or *Rag2*^{-/-} hosts (**Figure 7A**), although most env-reactive donor CD4⁺ T cells did not exhibit detectable CD25 expression in either type of host. As expression of CD25 may also be induced by IL-2 signaling, it was possible that, as well as reduced responsiveness of EF4.1 CD4⁺ T cells to IL-2, production or availability of IL-2 was reduced in WT hosts. Indeed, serum IL-2 was minimally detected during FV infection of lymphocyte-replete hosts, even when B6 mice with increased genetic susceptibility, provided by the *Fv2*^s allele (36), were used (**Figure 7B**). In contrast, transfer of EF4.1 CD4⁺ T cells into FV-infected lymphocyte-deficient *Rag1*^{-/-} hosts led to readily detectable serum IL-2 (**Figure 7C**).

IL-2 transcription was previously found to be reduced in env-specific EF4.1 CD4⁺ T cells responding to FV infection than to Ad5.pIX-gp70 immunization (37). However, relative reduction

of IL-2 transcription in FV infection could simply reflect the strong skewing toward Tfh cells, which may not produce IL-2. To further investigate the nature of IL-2-producing CD4⁺ T cells, we searched for correlates of IL-2 production in single-cell RNA-sequencing data obtained with env-specific EF4.1 CD4⁺ T cells primed by FV or Ad5.pIX-gp70 (39). This analysis revealed that most EF4.1 CD4⁺ T cells transcribing *Il2* in response to FV infection, also transcribed *Il21*, *Il10* or *Ifng* (**Figure 7D**). In contrast, transcription of distinct cytokine genes was largely restricted to different EF4.1 CD4⁺ T cells responding to Ad5.pIX-gp70 immunization, with most *Il2*-positive cells lacking transcripts for other cytokines, with the exception of *Gzmb* (**Figure 7D**). These data suggest that env-specific EF4.1 CD4⁺ T cells producing IL-2 following FV infection or Ad5.pIX-gp70 immunization display disparate functional properties. Together, these results highlighted the important contribution of reduced IL-2 production and availability to the dominant Tfh skewing in the CD4⁺ T cell response to FV infection.

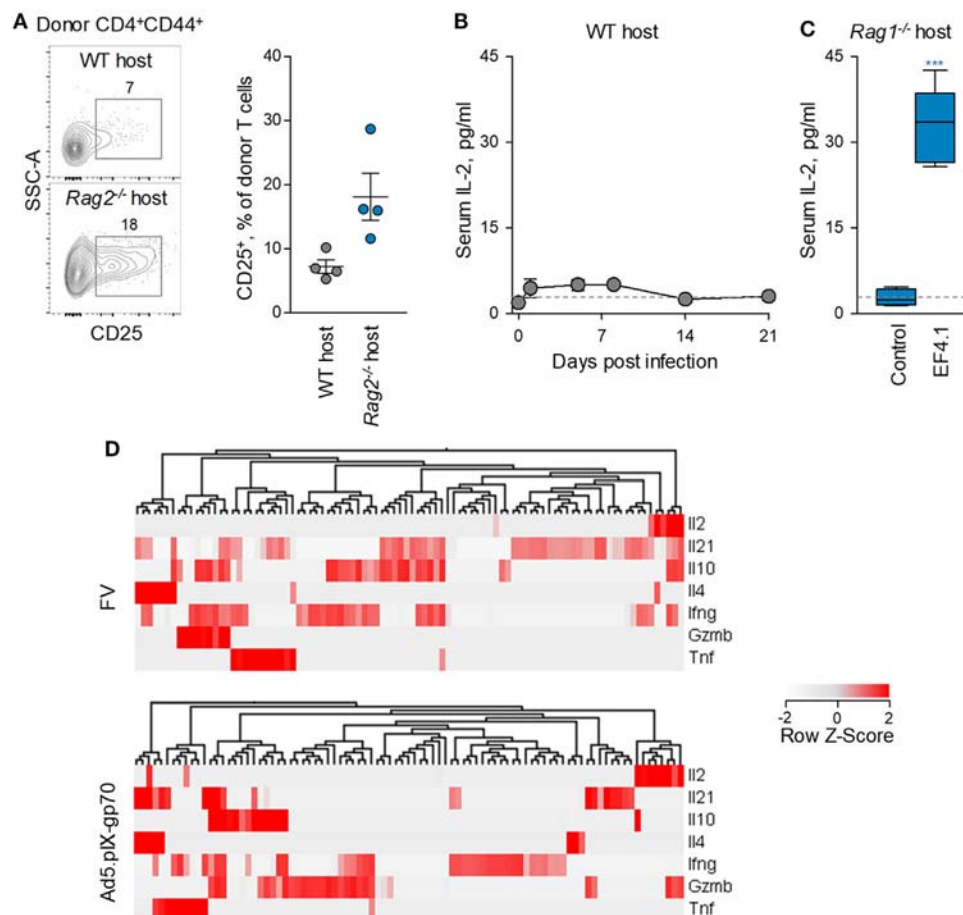


FIGURE 7 | Defective IL-2-IL-2R axis in CD4⁺ T cell response to FV-MLV env. **(A)** Flow cytometric example (*left*) and frequency of CD25 expressing cells (*right*) expression in env-reactive donor CD4⁺ T cells, 7 days after transfer into Friend virus (FV) infected WT or *Rag2*^{-/-} recipients. **(B)** Mean (±SEM) levels of IL-2 in the serum of WT B6.*Fv2*^s mice at different time-points after intermediate FV infection (*n* = 4–5 per time-point). **(C)** Mean (±SEM) levels of IL-2 in the serum of *Rag1*^{-/-} mice 35 days after infection with an intermediate dose of FV (control, *n* = 4) and those additionally received CD4⁺ EF4.1 T cells at the time of infection (EF4.1, *n* = 5). **(D)** Heatmap and hierarchical clustering of cytokine gene expression, assessed by single-cell RNA sequencing, in env-reactive donor CD4⁺ T cells isolated from the spleens of WT recipients infected with FV or immunized with Ad5.pIX-gp70. Each column represents an individual cell.

Distinct Correlates of Tfh and Th1 Differentiation in Response to FV Infection

To investigate possible correlates of Tfh bias in FV infection, we compared a number of variables relating to the strength of

TCR signaling. This was assessed independently by the degree of clonal expansion and resulting clonotypic composition, the degree of surface TCR downregulation, the activity of a Nur77-GFP reporter (**Figure 8A**) (31), and the degree of PD-1 and

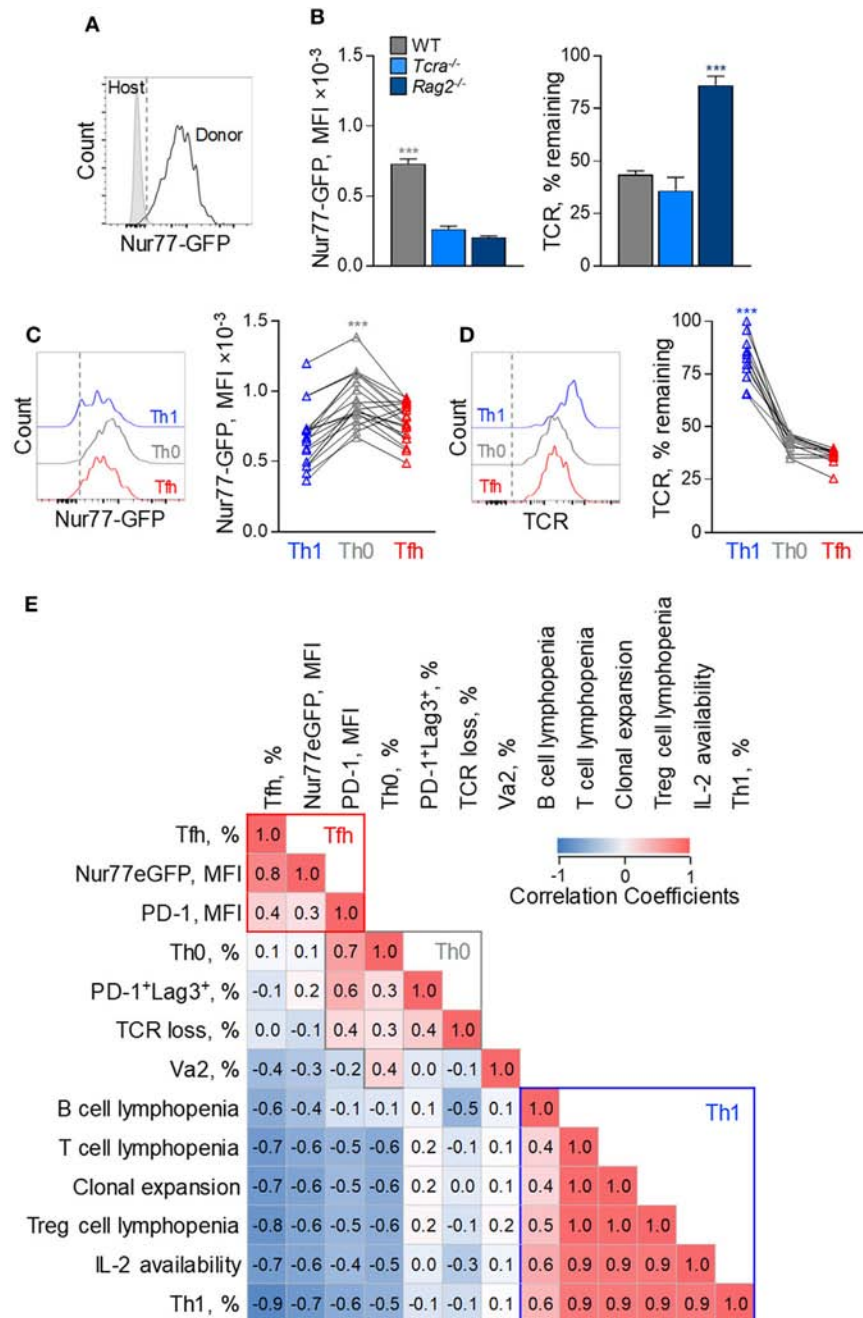


FIGURE 8 | Correlates of follicular helper cells (Tfh) and Th1 CD4⁺ T cell response to FV infection. **(A)** Representative flow cytometric profile of GFP expression in Nur77-GFP EF4.1 doubly transgenic env-reactive donor CD4⁺ T cells, 7 days post transfer into Friend virus (FV)-infected WT recipients. **(B)** Mean fluorescence intensity (MFI) ($\pm$ SEM) of Nur77-GFP expression (left) and levels of TCR β expression (right) in env-reactive donor CD4⁺ T cells, 7 days post transfer into WT ($n = 4$), *Tcr*^{-/-} ($n = 3$) or *Rag2*^{-/-} ($n = 4$) recipients, infected with an intermediate dose of FV. One representative of three experiments is shown. **(C)** Flow cytometric profile (left) and MFI of Nur77-GFP expression (right) and **(D)** flow cytometric profile (left) and levels of TCR β expression (right) in T helper (Th) subsets, defined by PSGL1 and CXCR5 expression, in env-reactive donor CD4⁺ T cells, 7 days after transfer into FV-infected WT recipients ($n = 19$). Lines connect values from individual recipients. **(E)** Matrix of correlation coefficients between the indicated variables and Th subset differentiation.

LAG3 expression. Although all these parameters can be directly affected by the strength of TCR signaling, their precise relationship or indeed their effect on Tfh differentiation is not necessarily linear.

For example, EF4.1 CD4⁺ T cells primed in WT hosts exhibited the strongest Nur77-GFP signals and highest degree of TCR downregulation, whereas those primed in *Rag2*^{-/-} hosts displayed the opposite phenotype (**Figure 8B**), indicating a direct correlation between Nur77-GFP intensity and TCR downregulation. However, EF4.1 CD4⁺ T cells primed in *Tcra*^{-/-} hosts downregulated their TCRs to the same degree as in WT hosts, but without the accompanying increase in Nur77-GFP reporter activity (**Figure 8B**). Also, partly discordant were the degree of TCR downregulation and Nur77-GFP reporter activity in Th functional subsets primed in the same host (**Figures 8C,D**). Th1 EF4.1 CD4⁺ T cells retained significant amounts of surface TCR, compared with Tfh or Th0 cells primed in WT hosts (**Figures 8C,D**), likely due to infrequent interaction of the former subset with B cells. However, Th0 cells exhibited significantly higher Nur77-GFP reporter activity, than either Th1 or Tfh cells in these hosts, despite comparable TCR downregulation with Tfh cells (**Figures 7C,D**). These results suggested that, although modulation of TCR signal strength is evidently different in EF4.1 CD4⁺ T cells primed in T cell-replete or T cell-deficient hosts, the effect of T cell lymphopenia on Th differentiation operated through additional mechanisms.

Given the complex patterns of correlation between independently measured variables and the degree of Tfh and Th1 differentiation, we next calculated a correlation matrix (**Figure 8E**). To this end, we assessed the relative contribution and possible interaction of 13 variables controlling Tfh differentiation measured in the 11 separate combinations of host and immunization or infection regimen described here. This analysis indicated three distinguishable clusters across all conditions, corresponding to each of the three major Th subsets observed in FV infection. Development of Tfh cells correlated most strongly with the activity of the Nur77-GFP reporter, taken to indicate the strength of signaling EF4.1 CD4⁺ T cells received, and also with the intensity of PD-1 expression (**Figure 8E**). In contrast, Th1 development exhibited strong anti-correlation with Nur77-GFP reporter activity and PD-1 expression levels and instead correlated with T cell lymphopenia and the availability of IL-2, together likely driving T cell clonal expansion (**Figure 8E**). Finally, uncommitted Th0 cells, although sharing many attributes with Tfh cells, appear to cluster separately, correlating strongly with LAG-3 expression, the degree of TCR downregulation and, the use of high-affinity TCRs (**Figure 8E**). Thus, our data suggest that Tfh differentiation is promoted by an optimal degree of TCR signaling, as well as by T cell competition.

DISCUSSION

It is now well recognized that both T cell-intrinsic and T cell-extrinsic factors shape the balance of CD4⁺ T cell differentiation into distinguishable functional subsets (1–8). However, the relative contribution of each of these factors in isolation or their potential intersection with each other in the context of diverse

immunological challenges is still not fully understood. Here, we provided evidence to suggest that the CD4⁺ T cell response to a model retroviral antigen, presented during natural infection, is heavily skewed toward Tfh differentiation. This allowed us to identify the variables that best correlate with the degree of Tfh differentiation in this model and to accurately quantify their contribution.

The variable that exhibited the closest positive correlation with Tfh differentiation of env-reactive CD4⁺ T cells in all the conditions studied was the strength of TCR signaling, translating to increased transcription of the Nur77-GFP reporter. Also, directly correlating with both the degree of Tfh differentiation and Nur77-GFP reporter activity was the intensity of PD-1 expression. These findings are consistent with an instructive model, whereby stronger TCR signaling in Th cell precursors favors Tfh development, likely through stronger induction of the Tfh-promoting cytokine IL-21 (42, 48, 49).

Although stronger TCR signaling in Tfh cells was indicated by the increase in Nur77-GFP reporter activity and the intensity of PD-1 expression, it should be noted that not all correlates of TCR signaling followed a similar pattern. For example, the degree of surface TCR downregulation, generally proportional to TCR signal strength (50), did not correlate with the degree of Tfh differentiation. Moreover, expression of LAG3, which is also transiently induced by strong TCR signals (51, 52), showed no positive correlation with Tfh differentiation. Instead, the env-reactive CD4⁺ T cells with the highest TCR signal, assessed by both the activity of the Nur77-GFP reporter and the degree of surface TCR downregulation, appeared inhibited in their commitment to either the Th1 or Tfh subsets. These cells, which we refer to as uncommitted Th0 cells (44), also strongly correlated with co-expression of the inhibitory receptors PD-1 and LAG3.

Seemingly uncommitted Th0 cells are not typically observed in acute viral infections (44), but a similar population lacking either Th1 or Tfh characteristics has been described in CD4⁺ T cells primed during the chronic phase of LCMV Cl13 infection (18). In that model, the emergence of Th0 cells was linked to an initial defect in effector differentiation, indirectly caused by chronic IFN type I production (18). Eventually, CD4⁺ T cells primed during chronic LCMV-specific developed almost exclusively into Tfh cells (18).

The env-reactive CD4⁺ T cells with Th0 characteristics observed during FV infection are also closely related to Tfh cells, highlighting parallels between acute FV infection and chronic LCMV infection. However, as IFN type I production is not a prominent feature of acute FV infection (40), defective effector differentiation must have alternative explanations. Our results with the FV model suggest that TCR signaling above an optimal strength restrains differentiation of env-reactive CD4⁺ T cells, which would otherwise be committed to the Tfh subset. Indeed, in addition to displaying the highest Nur77-GFP reporter activity and preferentially expressed genes involved in active metabolism, Th0 cells were most noticeable in conditions associated with the highest antigenic load or potency. Furthermore, the frequency of Th0 in env-reactive CD4⁺ T cells was positively correlated with the percentage of PD-1 and LAG3 coexpressing cells across the various conditions studied and was reduced by anti-PD-1 and

anti-LAG3 treatment during FV infection, which promoted their differentiation into Tfh cells. Notably, Tfh differentiation was also reported to be enhanced by anti-PD-1 and anti-LAG3 treatment during *Plasmodium yoelii* infection of mice (53), indicating that this pathway is restricting Tfh differentiation in persistent parasitic, as well as viral infection. Thus, our results suggest that Tfh differentiation is most efficiently induced by an optimal range of TCR signal strength.

Although evidently influenced by TCR signal strength, Tfh differentiation of the CD4⁺ T cell response to F-MLV env was independent of TCR clonotypic affinity, as previously suggested (37, 41, 42). Env-reactive CD4⁺ T cells bearing identical high-affinity clonotypic TCRs (identified by the use of endogenous Vα2 chains) were found both in the Tfh and Th1 subsets at comparable frequencies. Notably, despite comparable clonotypic TCR usage and Nur77-GFP reporter activity, Th1 env-reactive CD4⁺ T cells retained nearly the full amount of surface TCR, when compared with their Tfh counterparts in the same host, which had nearly lost their surface TCR expression. Therefore, the differential strength of TCR signals received by Th1 and Tfh cells cannot be solely attributed to differences in clonotypic TCR affinity. Instead, at least part of the differential TCR signaling between Th1 and Tfh cells is secondary to their differentiation and likely the result of their anatomic localization and interaction with distinct APC types. Supporting this notion, downregulation of surface TCR expression in CD4⁺ T cells has been previously shown, in FV infection (42), as well as in other model systems (54, 55), to be dependent on interaction with antigen-presenting B cells. Retention of surface TCR preferentially in Th1 cells would, therefore, suggest reduced B cell interaction. A central role for the APC type in determining or consolidating Th1 or Tfh effector differentiation may also underlie the propensity of different vaccine vectors or immunization regimens to induce distinct ratios of Th1 and Tfh response to a given antigen.

In addition to interaction with distinct APC types, our findings also support the concept that interaction and/or competition between T cells are also critical in determining effector differentiation, particularly, of Th1 cells. Although expectedly B cell deficiency did promote Th1 responses at the expense of Tfh responses, the strongest positive effect on Th1 differentiation was T cell lymphopenia. Indeed, the proportion of Th1 cells was approximately threefold higher in T cell deficiency than in B cell deficiency. Conditions conducive for Th1 differentiation in T cell lymphopenia are likely to involve deficiency in Treg cells, which can reduce availability of IL-2, thus promoting Tfh differentiation (24). However, an additional role for primary IL-2 production by effector CD4⁺ T cells was also indicated by

single-cell RNA-sequencing analysis. Although Th1-promoting *Il2* transcription was still detected in single CD4⁺ T cells despite the heavy skewing toward a Tfh response following FV infection, this was always accompanied by transcription of Th1-suppressing cytokines, such as *Il21* and *Il10*. In contrast, *Il2* transcription in single CD4⁺ T cells primed by Ad5.pIX-gp70 vaccination partly overlapped only with *Gzmb* transcription.

Together, our results highlight the potent contribution of T cell-extrinsic variables to determine the relative balance of Th1 and Tfh responses. Manipulating these variables in vaccination regimens in order to achieve a balance of CD4⁺ T cell effector differentiation appropriate for the respective context (e.g., viral infection or cancer) will be the next important challenge.

ETHICS STATEMENT

All animal experiments were approved by the ethical committee of the Francis Crick Institute, and conducted according to local guidelines and UK Home Office regulations under the Animals Scientific Procedures Act 1986 (ASPA).

AUTHOR CONTRIBUTIONS

LD and TD performed the experiments and analyzed the data. LD and GK wrote the manuscript. GK supervised the study and contributed to data analysis.

ACKNOWLEDGMENTS

We wish to thank Dr. Kristin A. Hogquist for the Nur77-GFP mice, Dr. Kim Hasenkrug for FV and FBL-3 stocks, and Dr. Ulf Dittmer for the Ad5.pIX-gp70 stocks. We are grateful for assistance from the Flow Cytometry and Biological Resource Facilities at the Francis Crick Institute.

FUNDING

This work was supported by the Francis Crick Institute, which receives its core funding from Cancer Research UK (FC001099), the UK Medical Research Council (FC001099), and the Wellcome Trust (FC001099).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at <https://www.frontiersin.org/articles/10.3389/fimmu.2018.01260/full#supplementary-material>.

REFERENCES

- O'shea JJ, Paul WE. Mechanisms underlying lineage commitment and plasticity of helper CD4⁺ T cells. *Science* (2010) 327(5969):1098–102. doi:10.1126/science.1178334
- Zhu J, Yamane H, Paul WE. Differentiation of effector CD4⁺ T cell populations. *Annu Rev Immunol* (2010) 28:445–89. doi:10.1146/annurev-immunol-030409-101212
- Crotty S. T follicular helper cell differentiation, function, and roles in disease. *Immunity* (2014) 41(4):529–42. doi:10.1016/j.immuni.2014.10.004
- Vinuesa CG, Linterman MA, Yu D, MacLennan IC. Follicular helper T Cells. *Annu Rev Immunol* (2016) 34:335–68. doi:10.1146/annurev-immunol-041015-055605
- Qi H. T follicular helper cells in space-time. *Nat Rev Immunol* (2016) 16(10):612–25. doi:10.1038/nri.2016.94
- Linterman MA, Vinuesa CG. Signals that influence T follicular helper cell differentiation and function. *Semin Immunopathol* (2010) 32(2):183–96. doi:10.1007/s00281-009-0194-z
- Lo WL, Allen PM. Self-awareness: how self-peptide/MHC complexes are essential in the development of T cells. *Mol Immunol* (2013) 55(2):186–9. doi:10.1016/j.molimm.2012.10.028

8. Thorborn G, Young GR, Kassiotis G. Effective T helper cell responses against retroviruses: are all clonotypes equal? *J Leukoc Biol* (2014) 96(1):27–37. doi:10.1189/jlb.2RI0613-347R
9. Choi YS, Gullicksrud JA, Xing S, Zeng Z, Shan Q, Li F, et al. LEF-1 and TCF-1 orchestrate T(FH) differentiation by regulating differentiation circuits upstream of the transcriptional repressor Bcl6. *Nat Immunol* (2015) 16(9):980–90. doi:10.1038/ni.3226
10. Gullicksrud JA, Li F, Xing S, Zeng Z, Peng W, Badovinac VP, et al. Differential requirements for Tcf1 long isoforms in CD8(+) and CD4(+) T cell responses to acute viral infection. *J Immunol* (2017) 199(3):911–9. doi:10.4049/jimmunol.1700595
11. Lee J, Hashimoto M, Im SJ, Araki K, Jin HT, Davis CW, et al. Adenovirus serotype 5 vaccination results in suboptimal CD4 T helper 1 responses in mice. *J Virol* (2017) 91(5):e1132–1116. doi:10.1128/jvi.01132-16
12. Shaw LA, Belanger S, Omilusik KD, Cho S, Scott-Browne JP, Nance JP, et al. Id2 reinforces TH1 differentiation and inhibits E2A to repress TFH differentiation. *Nat Immunol* (2016) 17(7):834–43. doi:10.1038/ni.3461
13. Wu T, Shin HM, Moseman EA, Ji Y, Huang B, Harly C, et al. TCF1 is required for the T follicular helper cell response to viral infection. *Cell Rep* (2015) 12(12):2099–110. doi:10.1016/j.celrep.2015.08.049
14. Xu L, Cao Y, Xie Z, Huang Q, Bai Q, Yang X, et al. The transcription factor TCF-1 initiates the differentiation of T(FH) cells during acute viral infection. *Nat Immunol* (2015) 16(9):991–9. doi:10.1038/ni.3229
15. Boettler T, Choi YS, Salek-Ardakani S, Cheng Y, Moeckel F, Croft M, et al. Exogenous OX40 stimulation during lymphocytic choriomeningitis virus infection impairs follicular Th cell differentiation and diverts CD4 T cells into the effector lineage by upregulating Blimp-1. *J Immunol* (2013) 191(10):5026–35. doi:10.4049/jimmunol.1300013
16. Lewis GM, Wehrens EJ, Labarta-Bajo L, Streeck H, Zuniga EI. TGF-beta receptor maintains CD4 T helper cell identity during chronic viral infections. *J Clin Invest* (2016) 126(10):3799–813. doi:10.1172/jci87041
17. Maine CJ, Teijaro JR, Marquardt K, Sherman LA. PTPN22 contributes to exhaustion of T lymphocytes during chronic viral infection. *Proc Natl Acad Sci U S A* (2016) 113(46):E7231–9. doi:10.1073/pnas.1603738113
18. Osokine I, Snell LM, Cunningham CR, Yamada DH, Wilson EB, Elsaesser HJ, et al. Type I interferon suppresses de novo virus-specific CD4 Th1 immunity during an established persistent viral infection. *Proc Natl Acad Sci U S A* (2014) 111(20):7409–14. doi:10.1073/pnas.1401662111
19. Snell LM, Osokine I, Yamada DH, De La Fuente JR, Elsaesser HJ, Brooks DG. Overcoming CD4 Th1 cell fate restrictions to sustain antiviral CD8 T cells and control persistent virus infection. *Cell Rep* (2016) 16(12):3286–96. doi:10.1016/j.celrep.2016.08.065
20. Ballesteros-Tato A, Leon B, Graf BA, Moquin A, Adams PS, Lund FE, et al. Interleukin-2 inhibits germinal center formation by limiting T follicular helper cell differentiation. *Immunity* (2012) 36(5):847–56. doi:10.1016/j.immuni.2012.02.012
21. Leon B, Bradley JE, Lund FE, Randall TD, Ballesteros-Tato A. FoxP3+ regulatory T cells promote influenza-specific Tfh responses by controlling IL-2 availability. *Nat Commun* (2014) 5:3495. doi:10.1038/ncomms4495
22. McDonald PW, Read KA, Baker CE, Anderson AE, Powell MD, Ballesteros-Tato A, et al. IL-7 signalling represses Bcl-6 and the TFH gene program. *Nat Commun* (2016) 7:10285. doi:10.1038/ncomms10285
23. Read KA, Powell MD, Baker CE, Sreekumar BK, Ringel-Scaia VM, Bachus H, et al. Integrated STAT3 and Ikaros zinc finger transcription factor activities regulate Bcl-6 expression in CD4(+) Th cells. *J Immunol* (2017) 199(7):2377–87. doi:10.4049/jimmunol.1700106
24. Botta D, Fuller MJ, Marquez-Lago TT, Bachus H, Bradley JE, Weinmann AS, et al. Dynamic regulation of T follicular regulatory cell responses by interleukin 2 during influenza infection. *Nat Immunol* (2017) 18(11):1249–60. doi:10.1038/ni.3837
25. Li J, Lu E, Yi T, Cyster JG. EBI2 augments Tfh cell fate by promoting interaction with IL-2-quenching dendritic cells. *Nature* (2016) 533(7601):110–4. doi:10.1038/nature17947
26. Ray JP, Staron MM, Shyer JA, Ho PC, Marshall HD, Gray SM, et al. The interleukin-2-mTORC1 kinase axis defines the signaling, differentiation, and metabolism of T helper 1 and follicular B helper T cells. *Immunity* (2015) 43(4):690–702. doi:10.1016/j.immuni.2015.08.017
27. Weinstein JS, Bertino SA, Hernandez SG, Poholek AC, Teplitzky TB, Nowyhed HN, et al. B cells in T follicular helper cell development and function: separable roles in delivery of ICOS ligand and antigen. *J Immunol* (2014) 192(7):3166–79. doi:10.4049/jimmunol.1302617
28. Hasenkrug KJ, Chesebro B. Immunity to retroviral infection: the friend virus model. *Proc Natl Acad Sci U S A* (1997) 94(15):7811–6. doi:10.1073/pnas.94.15.7811
29. Tsuji-Kawahara S, Kawabata H, Matsukuma H, Kinoshita S, Chikaishi T, Sakamoto M, et al. Differential requirements of cellular and humoral immune responses for Fv2-associated resistance to erythroleukemia and for regulation of retrovirus-induced myeloid leukemia development. *J Virol* (2013) 87(24):13760–74. doi:10.1128/JVI.02506-13
30. Antunes I, Tolaini M, Kissenpfennig A, Iwashiro M, Kuribayashi K, Malissen B, et al. Retrovirus-specificity of regulatory T cells is neither present nor required in preventing retrovirus-induced bone marrow immune pathology. *Immunity* (2008) 29(5):782–94. doi:10.1016/j.immuni.2008.09.016
31. Moran AE, Holzapfel KL, Xing Y, Cunningham NR, Maltzman JS, Punt J, et al. T cell receptor signal strength in Treg and iNKT cell development demonstrated by a novel fluorescent reporter mouse. *J Exp Med* (2011) 208(6):1279–89. doi:10.1084/jem.20110308
32. Kitamura D, Roes J, Kuhn R, Rajewsky K. A B cell-deficient mouse by targeted disruption of the membrane exon of the immunoglobulin mu chain gene. *Nature* (1991) 350(6317):423–6. doi:10.1038/350423a0
33. Philpott KL, Viney JL, Kay G, Rastan S, Gardiner EM, Chae S, et al. Lymphoid development in mice congenitally lacking T cell receptor alpha beta-expressing cells. *Science* (1992) 256(5062):1448–52. doi:10.1126/science.1604321
34. Mombaerts P, Iacomini J, Johnson RS, Herrup K, Tonegawa S, Papaioannou VE. RAG-1-deficient mice have no mature B and T lymphocytes. *Cell* (1992) 68(5):869–77. doi:10.1016/0092-8674(92)90030-G
35. Monroe RJ, Seidl KJ, Gaertner F, Han S, Chen F, Sekiguchi J, et al. RAG2:GFP knockin mice reveal novel aspects of RAG2 expression in primary and peripheral lymphoid tissues. *Immunity* (1999) 11(2):201–12. doi:10.1016/S1074-7613(00)80095-3
36. Marques R, Antunes I, Eksmond U, Stoye J, Hasenkrug K, Kassiotis G. B lymphocyte activation by coinfection prevents immune control of friend virus infection. *J Immunol* (2008) 181(5):3432–40. doi:10.4049/jimmunol.181.5.3432
37. Thorborn G, Ploquin MJ, Eksmond U, Pike R, Bayer W, Dittmer U, et al. Clonotypic composition of the CD4+ T cell response to a vectored retroviral antigen is determined by its speed. *J Immunol* (2014) 193(4):1567–77. doi:10.4049/jimmunol.1400667
38. Klarnet JP, Kern DE, Okuno K, Holt C, Lilly F, Greenberg PD. FBL-reactive CD8+ cytotoxic and CD4+ helper T lymphocytes recognize distinct friend murine leukemia virus-encoded antigens. *J Exp Med* (1989) 169(2):457–67. doi:10.1084/jem.169.2.457
39. Donnarumma T, Young GR, Merkenschlager J, Eksmond U, Bongard N, Nutt SL, et al. Opposing development of cytotoxic and follicular helper CD4 T cells controlled by the TCF-1-Bcl6 Nexus. *Cell Rep* (2016) 17(6):1571–83. doi:10.1016/j.celrep.2016.10.013
40. Duley AK, Ploquin MJ, Eksmond U, Ammann CG, Messer RJ, Myers L, et al. Negative impact of IFN-gamma on early host immune responses to retroviral infection. *J Immunol* (2012) 189(5):2521–9. doi:10.4049/jimmunol.1201125
41. Merkenschlager J, Ploquin MJ, Eksmond U, Andargachew R, Thorborn G, Filby A, et al. Stepwise B-cell-dependent expansion of T helper clonotypes diversifies the T-cell response. *Nat Commun* (2016) 7:10281. doi:10.1038/ncomms10281
42. Ploquin MJ, Eksmond U, Kassiotis G. B cells and TCR avidity determine distinct functions of CD4+ T cells in retroviral infection. *J Immunol* (2011) 187(6):3321–30. doi:10.4049/jimmunol.1101006
43. Marshall HD, Chandele A, Jung YW, Meng H, Poholek AC, Parish IA, et al. Differential expression of Ly6C and T-bet distinguish effector and memory Th1 CD4(+) cell properties during viral infection. *Immunity* (2011) 35(4):633–46. doi:10.1016/j.immuni.2011.08.016
44. Crotty S. Do memory CD4 T cells keep their cell-type programming: plasticity versus fate commitment? Complexities of interpretation due to the heterogeneity of memory CD4 T cells, including T follicular helper cells. *Cold Spring Harb Perspect Biol* (2018) 10(3):a032102. doi:10.1101/cshperspect.a032102
45. Choi YS, Yang JA, Yusuf I, Johnston RJ, Greenbaum J, Peters B, et al. Bcl6 expressing follicular helper CD4 T cells are fate committed early and have the capacity to form memory. *J Immunol* (2013) 190(8):4014–26. doi:10.4049/jimmunol.1202963

46. Young GR, Ploquin MJ, Eksmond U, Wadwa M, Stoye JP, Kassiotis G. Negative selection by an endogenous retrovirus promotes a higher-avidity CD4+ T cell response to retroviral infection. *PLoS Pathog* (2012) 8(5):e1002709. doi:10.1371/journal.ppat.1002709
47. Bayer W, Tenbusch M, Lietz R, Johrden L, Schimmer S, Uberla K, et al. Vaccination with an adenoviral vector that encodes and displays a retroviral antigen induces improved neutralizing antibody and CD4+ T-cell responses and confers enhanced protection. *J Virol* (2010) 84(4):1967–76. doi:10.1128/JVI.01840-09
48. Fazilleau N, Mcheyzer-Williams LJ, Rosen H, Mcheyzer-Williams MG. The function of follicular helper T cells is regulated by the strength of T cell antigen receptor binding. *Nat Immunol* (2009) 10(4):375–84. doi:10.1038/ni.1704
49. Nurieva RI, Podd A, Chen Y, Alekseev AM, Yu M, Qi X, et al. STAT5 protein negatively regulates T follicular helper (Tfh) cell generation and function. *J Biol Chem* (2012) 287(14):11234–9. doi:10.1074/jbc.M111.324046
50. Lanzavecchia A, Sallusto F. Antigen decoding by T lymphocytes: from synapses to fate determination. *Nat Immunol* (2001) 2(6):487–92. doi:10.1038/88678
51. Anderson AC, Joller N, Kuchroo VK. Lag-3, Tim-3, and TIGIT: co-inhibitory receptors with specialized functions in immune regulation. *Immunity* (2016) 44(5):989–1004. doi:10.1016/j.immuni.2016.05.001
52. Andrews LP, Marciscano AE, Drake CG, Vignali DA. LAG3 (CD223) as a cancer immunotherapy target. *Immunol Rev* (2017) 276(1):80–96. doi:10.1111/imr.12519
53. Butler NS, Moebius J, Pewe LL, Traore B, Doumbo OK, Tygrett LT, et al. Therapeutic blockade of PD-L1 and LAG-3 rapidly clears established blood-stage *Plasmodium* infection. *Nat Immunol* (2011) 13(2):188–95. doi:10.1038/ni.2180
54. Han S, Asoyan A, Rabenstein H, Nakano N, Obst R. Role of antigen persistence and dose for CD4+ T-cell exhaustion and recovery. *Proc Natl Acad Sci U S A* (2010) 107(47):20453–8. doi:10.1073/pnas.1008437107
55. Knoechel B, Lohr J, Kahn E, Abbas AK. The link between lymphocyte deficiency and autoimmunity: roles of endogenous T and B lymphocytes in tolerance. *J Immunol* (2005) 175(1):21–6. doi:10.4049/jimmunol.175.1.21

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2018 Danelli, Donnarumma and Kassiotis. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



Unexpected Help: Follicular Regulatory T Cells in the Germinal Center

Markus M. Xie and Alexander L. Dent*

Department of Microbiology and Immunology, Indiana University School of Medicine, Indianapolis, IN, United States

OPEN ACCESS

Edited by:

Georgia Foustari,
San Raffaele Hospital
(IRCCS), Italy

Reviewed by:

Luis Graca,
Universidade de Lisboa,
Portugal
Talal A. Chatila,
Harvard University,
United States

*Correspondence:

Alexander L. Dent
adent2@iupui.edu

Specialty section:

This article was submitted
to T Cell Biology,
a section of the journal
Frontiers in Immunology

Received: 20 April 2018

Accepted: 21 June 2018

Published: 02 July 2018

Citation:

Xie MM and Dent AL (2018)
Unexpected Help: Follicular
Regulatory T Cells in the
Germinal Center.
Front. Immunol. 9:1536.
doi: 10.3389/fimmu.2018.01536

Follicular helper T (T_{fh}) cells are necessary for germinal center (GC) formation and within the GC, provide key signals to B cells for their differentiation into plasmablasts and plasma cells that secrete high-affinity and isotype-switched antibody (Ab). A specialized subset of Foxp3⁺ T cells termed T follicular regulatory (T_{fr}) cells, also regulate the differentiation of Ab-secreting cells from the GC. T_{fr}-cell function in the GC is not well understood, however, the dominant paradigm currently is that T_{fr} cells repress excessive T_{fh} and GC B cell proliferation and help promote stringent selection of high-affinity B cells. A mouse model where the Bcl6 gene is specifically deleted in Foxp3⁺ T cells (Bcl6^{FC} mice) allows the study of T_{fr} cell function with more precision than other approaches. Studies with this model have shown that T_{fr} cells play a key role in maintaining GC B cell proliferation and Ab levels. Part of the mechanism for this positive “helper” effect of T_{fr} cells on the GC is T_{fr} cell-derived IL-10, which can promote B cell growth and entry into the dark zone of the GC. Recent studies on T_{fr} cells support a new paradigm for T_{fr} cell function in the GC reaction. Here, we review studies on T_{fr} cell functions and discuss the evidence that T_{fr} cells can have a major helper role in the GC-dependent Ab response.

Keywords: T follicular regulatory cells, germinal center, follicular helper T cell, regulatory T cells, T cell differentiation

INTRODUCTION

A major function of the adaptive immune response is to produce highly specific antibodies (Abs) that bind to antigen (Ag) with high affinity and help to eliminate pathogens and foreign substances. A specialized subset of differentiated CD4 T cells, follicular helper T (T_{fh}) cells, are required in the germinal center (GC) reaction to help B cells generate high-affinity Abs to Ag (1, 2). T_{fh} cells control the initiation as well as the outcome of the GC B cell response (3–6). T_{fh} cells are critical for the proper production of protective Abs during an infection, however, the over-production of T_{fh} cells can also lead to autoimmunity since T_{fh} cells can help B cells to produce self-reactive Abs (6–8). Thus, the proper regulation of T_{fh} cell differentiation is essential both for normal immune function and for preventing autoimmune disease.

Germinal center B cell responses are also regulated by T follicular regulatory (T_{fr}) cells, which develop from regulatory T cells (Tregs) and localize to the GC (9–16) (Figure 1). T_{fr} cells are generally thought to limit the function of T_{fh} cells in the GC (9–13, 16). T_{fr} cells, like T_{fh} cells, are dependent upon the transcriptional repressor protein Bcl6 for their development, but unlike T_{fh} cells express the canonical Treg master regulatory transcription factor Foxp3 (9–16). The prevailing model for T_{fr} cell function currently is that T_{fr} cells repress excessive T_{fh} and GC B cell proliferation and promote the selection of high-affinity B cells (9–13, 16), however, the complete range of T_{fr} cell functions are poorly understood. A T_{fr}-deficient mouse model where the Bcl6 gene is specifically deleted in Foxp3⁺ T cells (Bcl6^{fl/fl} Foxp3-Cre or Bcl6^{FC} mice) has been used by us and others to study T_{fr} cell function

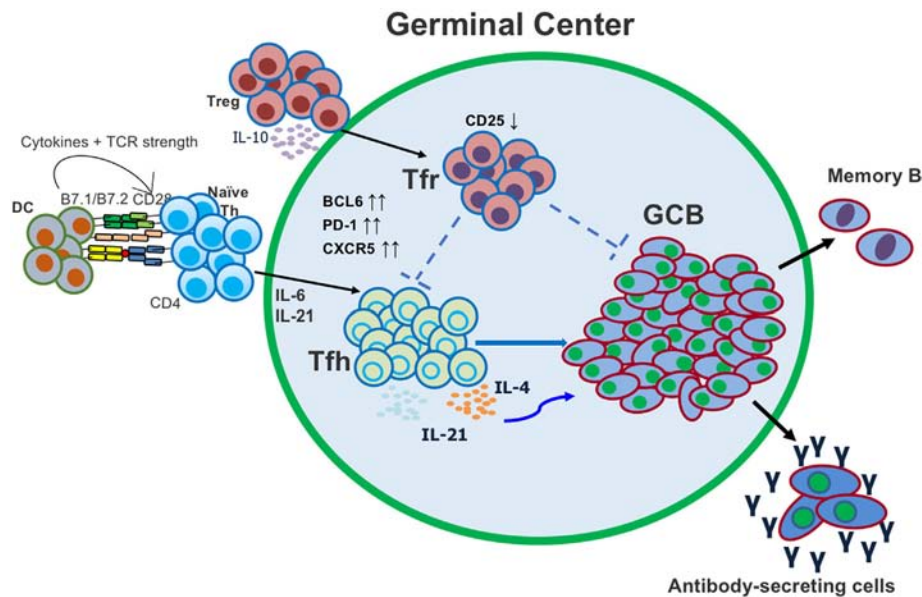


FIGURE 1 | Follicular helper T (Tfh) and T follicular regulatory (Tfr) cells both act in the germinal center (GC) to regulate the generation of antigen (Ag)-specific antibody-secreting cells. Tfh cells differentiate from conventional CD4 T cells after activation with Ag and dendritic cell (DC) presentation. Tfr cells differentiate from conventional regulatory T cells (Tregs) and migrate into the GC.

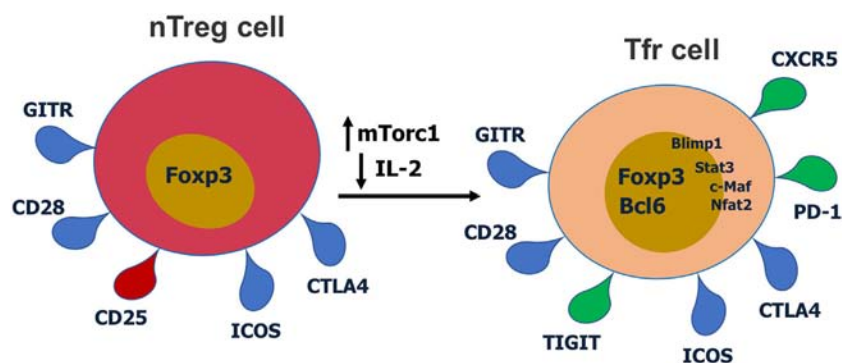


FIGURE 2 | Cell surface receptors and transcription factors involved in T follicular regulatory (Tfr) cell differentiation and function. Red receptors are downregulated and green receptors are upregulated during Tfr cell differentiation.

in immune and autoimmune responses (14, 17–19). With this model, we and others have unexpectedly found that the major Tfr cell function may not be inhibiting the GC response but instead helping promote the Ab response and even the magnitude of the GC response. Here, we discuss the current understanding of the differentiation and physiological functions of Tfr cells. We also discuss how Tfr cells balance suppressive and helper functions, the potential mechanisms underlying Tfr cell functions, and directions for future investigations.

DIFFERENTIATION AND REGULATION OF Tfr CELLS

Several studies have shown that Tfr cells primarily differentiate from Foxp3⁺ Treg precursor cells (10, 11, 20–22) (**Figure 2**),

however, like Tfh cells, Tfr cells can also develop from naïve CD4 T cells (23). Tregs are generated either during T cell differentiation in the thymus (tTregs) or from mature CD4 T cells in the periphery (pTregs) (24, 25), but whether Tfr cells preferentially develop from tTregs or pTregs is not known. Tregs in the intestinal mucosa are predominantly pTregs that develop to Ags derived from microbiota and diet as a tolerance mechanism (24, 25). Tfr cells that develop in the gut lymphoid tissues such as Peyer's patches may therefore differentiate from pTregs, and so ultimately may have a naïve CD4 T cell origin. Interestingly, Peyer's patch Tfr cells have a markedly different transcriptome than peripheral lymph node Tfr cells, possibly suggesting a different origin (26).

T follicular regulatory cells express Tfh cell surface markers such as PD-1, CXCR5, and ICOS, Treg surface markers such as CTLA-4 and GITR, and the master regulatory transcription

factors for both Tregs (Foxp3) and Tfh cells (Bcl6) (9–11, 14, 27). Thus, Tfr cells display a hybrid or mixed Tfh/Treg phenotype. Most studies have analyzed Tfr cells in the mouse, but phenotypically similar Tfr cells have also been described for humans (28, 29) and macaques (30). Tfr cells also express significant levels of Blimp1, a transcriptional repressor protein that suppresses Bcl6 expression (10, 31). Notably, Tfr cells express lower CD25 compared with non-Tfr Tregs (14, 29). Together with Bcl6, Nfat2 upregulates CXCR5 expression on Tregs and enables them to migrate to GC, take on the follicular phenotype and become Tfr cells (9–11, 14, 32). Recent work has revealed that the mTor pathway is a key regulator of Tfr cells. The mTorC1 complex is essential in regulating the conversion of Tregs to Tfr cells and this is potentially through a Stat3–Tcf-1–Bcl6 pathway (33, 34). Our lab has also specifically found that in contrast to Tfh cells which can develop in the absence of Stat3, Stat3 is essential for Tfr cell development (15). Deletion of *Pten* in Tregs leads to upregulated mTorC2 activity and heightened Tfr cell development (35). Thus, the Akt–mTor2 kinase pathway promotes Tfr cell development and the Pten phosphatase helps restrain excessive Tfr cell development (35).

Antigen exposure triggers the differentiation of Tfr cells and this process is dendritic cell (DC)-dependent (10, 11, 23, 27). Sage et al. used mice that express diphtheria toxin receptor specifically on DCs to test this (12). DC-depletion led to substantially decreased Tfr cells, however, it is unknown which specific DC subsets directly contribute to Tfr cell differentiation. At the same time, PD-1-ligand expressed on DCs has an inhibitory role on Tfr cell development (36). Tregs can repress the function of Ag presenting cells (APCs) including DCs (37), but whether Tfr cells can affect DCs or other APCs and how this might affect the GC response is unknown. Precisely what Ags and signals that Tregs respond to in order to become Tfr cells is not well understood. Tfr cells respond more strongly to self-Ags than foreign Ags, which fits with the self-reactive nature of tTregs (23, 38). While Tfr cells can be found that have specificity for the immunizing Ag (23), a recent study on the TCR specificity of Tfh and Tfr cells indicated that in contrast to Tfh cells, Tfr cells do not respond well to the cognate Ag after immunization (22). Furthermore, an analysis of TCR gene sequences in Tfh and Tfr cells indicated that Tfh cells are a sub-population of cells related to naïve CD4 T cells, whereas Tfr cells showed a TCR profile very similar to the total Treg population (22). These findings are consistent with the model that Tfh cells are Ag-specific T cells that proliferated after Ag stimulation, while Tfr cells develop in a polyclonal and Ag-independent manner from Tregs. Therefore, Tfr cells either develop from Tregs in a polyclonal TCR-dependent response involving recognition of self-Ag, or Tfr cells expand and differentiate by an Ag-independent and TCR independent pathway [e.g., Jagged1 plus Ox40 stimulation (39)]. Note that the Maceiras et al. study (22) of Tfr cell TCR sequences analyzed Tfr cells from peripheral LNs, and the TCR specificity of Peyer's patch Tfr cells may be more similar to naïve CD4 T cells that are responsive to gut Ags.

T cell co-stimulation is required for Tfr cell differentiation as either CD28 or ICOS deficiency leads to reduction of Tfr cells (10, 27, 40). Mice with CD28 deficiency specifically in Tregs

(using Foxp3-cre) had a large reduction in Tfr cells in the draining lymph node after NP-OVA immunization (40). This is largely due to the roles of CD28 in inducing Foxp3 expression as well as Tfr cell proliferation (10, 41–44). Similarly, Tfr cell development is abrogated in ICOS-deficient mice (27). ICOS signaling modulates the expression of Bcl6 and c-Maf in Tfh cells and might play a similar role in Tfr cells (45–47). Bcl6 is an essential transcription factor for Tfr cells, and recent studies suggest that c-Maf is also pivotal for Tfr cell differentiation (10, 11, 14, 48, 49). Bcl6 and Blimp1 reciprocally repress expression of the other factor in both Tfh and Tfr cells (31, 50). The regulation of Tfh cell differentiation by Blimp1 is Bcl6-dependent while Blimp1 controls Tfr cell differentiation independent of Bcl6 (31). One mechanism for Bcl6-independent Blimp1 activity may relate to regulation of Nfat2, which has been shown to be important for upregulation of CXCR5 on Tfr cells as well as for expression of PD-1 (32, 51). Blimp1 has been shown to repress Nfat2 expression (51), and thus Blimp1 could have a suppressive role for CXCR5 and PD-1, both of which are key genes increased in Tfr cells. Increased expression of Nfat2 in Blimp1-deficient Tregs could then lead to Bcl6-independent expression of CXCR5 and PD-1, and appearance of Tfr-like cells (31). Tfr cells were repressed by high IL-2 levels at the peak of influenza infection and this was through a Blimp1-dependent mechanism (19). IL-2 is also a negative signal for Bcl6 expression, and decreased IL-2 promotes induction of Tfr cells. After the peak anti-flu virus immune response, CD25 expression is downregulated in some Tregs while Bcl6 is increased, leading to Tfr programming (19). Thus, IL-2 is a key factor regulating Tfr differentiation, promoting Blimp1 expression while repressing Bcl6 in Tregs to preclude Tfr cell development.

PD-1, which is expressed by both Tfh and Tfr cells, inhibits Tfr differentiation and their suppressive function (10, 11, 16, 27). Sage et al. showed that Tfr cells in *Pdcd1*-deficient mice had greater suppressive function and resulted in decreased Ab production both *in vitro* and *in vivo* (27). The exact mechanism for the increased inhibitory function of *Pdcd1*-deficient Tfr cells remains unclear. At the same time, PD-1 ligand is required for Tfr cell generation, however, it is not clear if this is a direct or indirect effect on Tfr cells (23). Similarly, CTLA-4, the inhibitory receptor which binds to CD80 and CD86, limits the differentiation of Tfr cells (13, 52, 53). However, restricted CTLA-4 deficiency in Tregs contributes not only to enhanced Tfr cells but also enhanced Tfh, GCB cells, and Ab responses (53). One explanation is that in the absence of CTLA-4 function in Tregs, there is uncontrolled inflammation that drives higher Tfh cell and GCB responses. However, since it is not clear what drives the enhanced Tfh, GCB, and IgE responses, a “helper” role of Tfr cells cannot be completely excluded (53). Deletion of CTLA-4 results in increased IL-10 production by Tregs (54). Since IL-10 can promote GC responses (17, 55), it is possible that increased IL-10 production by Tfr cells contributes to the increased GC and Ab response in CTLA-4 KO mice.

The majority of research on Tfr cells has been conducted in the mouse system but a few recent studies have elucidated Tfr cell populations in human GCs that are basically similar to Tfr cells in mice (29, 56, 57). CXCR5⁺ Tfh-like cells in blood, also

known as circulating Tfh (cTfh) cells, are typically used as a proxy marker for the GC Tfh cell response in humans. By assessing cTfh cell frequency in patients with monogenic mutations leading to immunodeficiency, a large number of genes controlling human Tfh cell development have been categorized (58). Circulating Tfr (cTfr) cells in blood are also used as a correlate of the Tfr cell response (28, 59–62), however, in contrast to Tfh cells (58), relatively few genes that control Tfr cell development and function in humans have been characterized to date (e.g., *LRBA* and *CTLA4*) (62–64). Thus, much work remains in fully understanding specific genes and pathways that regulate human Tfr cells.

SUPPRESSIVE FUNCTIONS OF Tfr CELLS

T follicular regulatory cells have been described in the literature mainly as suppressors of the GC reaction and the Ab response, repressing the proliferation of Tfh cells and GC B cells, and limiting the generation of Ab-secreting cells and overall Ab responses. However, the experimental approaches taken in many studies can give rise to alternative interpretations. *In vitro*, Tfr cells can suppress the proliferation and cytokine production of Tfh cells as well as the proliferation and Ig secretion of B cells, similar to the *in vitro* suppressive function seen with non-Tfr Tregs (13, 27–29, 38, 65). *In vivo* studies have demonstrated that Tfr cells, analyzed initially by depletion of total Tregs, can suppress the numbers of GC B cells and Tfh cells (9–11, 13, 27, 53). However, these studies may not represent specific effects of Tfr cell depletion or physiological Tfr cell function. Total Treg deletion (10, 11, 13, 66) provokes severe inflammation and causes a very broad effect on T cell responses, thus obscuring the specific functions of Tfr cells. Studies using adoptive transfer of Tfr cells along with other T cells into T cell-deficient mice or Tfh-cell-deficient mice might have non-physiological effects due to the abnormal immune environment of the recipient mice (10, 11, 27, 33). Studies where Tfr cell numbers are greatly enhanced due to deletion of Roquin (34), or where Tregs are forced to migrate to the B cell follicle by ectopic CXCR5 expression (32) might also lead to non-physiological suppression and/or non-specific suppression of GC responses. Mice with the *Nfat2* gene deleted in Tregs with Foxp3-cre showed a partial loss of Tfr cells and augmented numbers of GC B cells, Tfh cells, and Ag-specific Abs after immunization (32). However, a more general loss of Treg function by loss of *Nfat2* affecting Tfh cell expansion cannot be discriminated from the specific effects from loss of Tfr cells. *In vitro* studies of Tfr cells cannot mimic the complex *in vivo* environment of the GC reaction and cannot analyze affinity selection of GC B cells. Together, a re-interpretation of the Tfr cell literature helps to explain why the function of Tfr cells assessed using Bcl6FC mice (14, 17, 19), is strikingly different from many other studies on Tfr cell function.

Nonetheless, it is clear that under some conditions, Tfr cells can negatively regulate the GC reaction, and the precise mechanisms that Tfr cells use to negatively regulate the GC is one of the unsolved mysteries in the Tfr cell field. Tregs can suppress immune responses by multiple known mechanisms: IL-2 consumption, secretion of inhibitory factors (IL-10, TGF- β , IL-35, granzyme B, CD39, CD73, and TRAIL), and CTLA-4-mediated

inhibition of Tfh cell co-stimulation (67–69). Of these known suppressor factors, we can narrow down mechanisms for Tfr cells based on previous data. Tfh cell differentiation is inhibited by IL-2 (70–72), and IL-2 consumption by Tfr cells could be predicted to help stabilize Tfh cell responses. However, Tfr cells have low levels of the high-affinity IL-2 receptor CD25 (14), which indicates a lessened capacity to compete for available IL-2. IL-10 is unlikely to be the key suppressor factor, since IL-10 is a stimulatory or growth factor for GC B cells (17), and furthermore, IL-10 expression by Tfh cells is increased in the absence of Tfr cells in Bcl6 mice (14). IL-35 is unlikely to be a Tfr suppressor factor as it primarily affects T cell proliferation (73), and data with Bcl6FC mice does not indicate an effect of Tfr cells on the number of Tfh cells (14). Granzyme B is unlikely as a major mechanism as it is decreased in Tfr cells compared to Tregs (10). Metabolic suppressor pathways such as CD39 and CD73 have not been extensively characterized and are possible effectors of suppression by Tfr cells as they could potentially affect cell proliferation in the GC. In mice, TGF- β is known to stabilize Tfh cell responses (74), and prevent excess Tfh cell responses (75). In humans, TGF- β is required for Tfh cell differentiation (76). A lack of TGF- β signaling from loss of Tfr cells does not clearly explain the normal Tfh cell numbers in the presence of increased Tfh cytokine expression in Bcl6FC mice (14). TRAIL is cytotoxic to follicular B cell lymphomas, which have a GC phenotype (77), but otherwise there is no data about TRAIL activity in GCs, particularly regarding Tfr cells. CTLA-4 expression by Tfr cells may inhibit the ability of Tfh cells to receive key co-stimulation signals from GC B cells, thus limiting Tfh cell and thus Tfh cell-driven GC B cell expansion. Unfortunately, studies on the role of CTLA-4 function in Tfr cells are difficult to interpret, as noted above (13, 52, 53).

A recently described mechanism for Tfr cells to inhibit Tfh cells and the GC is secretion of a decoy IL-1 receptor that inhibits Tfh cell differentiation (38). This pathway appears to be most critical during early Tfh cell activation and differentiation rather than during the GC reaction itself. Furthermore, data pointing to this decoy IL-1 receptor pathway being used specifically by Tfr cells to control Tfh cells *in vivo* is lacking. Another potential pathway used by Tfr cells to control Tfh cells and GC B cells is the inhibitory receptor TIGIT, that is important for Treg suppressive function (14, 78). Intriguingly, the two major suppressive pathways utilized by TIGIT^{high} Tregs are IL-10 and Fibrinogen-like protein 2 (Fgl2) (78). Fgl2 is a secreted protein that binds the inhibitory IgG receptor Fc γ RIIB (79). As noted above, it is unlikely that loss of IL-10 from Tfr cells contributes to the deregulated cytokine expression of Tfh cells. Thus, Fgl2 may be a key factor used by Tfr cells to regulate GC B cells. Interestingly, Fc γ RIIB KO mice are known to develop lupus (80), and Fgl2 KO mice develop glomerulonephritis, a pathologic manifestation of auto-Abs in severe lupus disease also seen in IgA nephropathy (81, 82). Fgl2 KO mice have Treg defects, but the GC response in these mice has not been characterized (81). TIGIT^{high} Tregs can also affect cell activation by inducing tolerogenic DCs *via* CD155 (83). But currently, data showing Tfr cells controlling the GC *via* TIGIT is lacking.

In analyzing mice with deletions of Bcl6 or Stat3 specifically in the Treg lineage (Bcl6FC and Stat3FC mice, respectively), we

found noteworthy differences between how Tfr cells are regulated by Stat3 and Bcl6 (14, 15). While Tfr cells are strongly depleted in both Bcl6FC and Stat3FC mice (14, 15), there are significant differences in the phenotype. First, in Bcl6FC but not Stat3FC mice, Tfh cells produce higher levels of cytokines compared to control mice. Second, Ag-specific IgA is increased in Bcl6FC mice whereas Ag-specific IgG is increased in Stat3FC mice (14, 15). At the same time, Tfh cell and GC B cell numbers are not altered in either Bcl6FC or Stat3FC mice compared to control mice (14, 15). The function of Stat3 in Tfr cells is not understood. Analogous to Tfh cells (84, 85), Stat3 may be important for Tfr cell development by inducing Bcl6 expression in Tregs in response to cytokines such as IL-6 and IL-21. Stat3 expression is also activated in Tfr cells by the mTorC1 pathway (33). Bcl6 is required for the development of the CXCR5⁺PD-1⁺ follicular T cell phenotype, and the induction of Bcl6 by STAT factors may be essential for both Tfr cell as well as Tfh cell development. If this is the case though, why does deletion of Stat3 in Foxp3-expressing cells produce a different phenotype than deletion of Bcl6 in Foxp3-expressing cells? Why are cytokines upregulated from Tfh cells in Bcl6FC mice but not in Stat3FC mice? The answer to this question is currently unknown but is essential for fully understanding Tfr cell development and function. One possible explanation for the difference is that there is a larger population of residual Tfr cells in Stat3FC mice compared to Bcl6FC mice and these residual Tfr cells in Stat3FC mice are enough to negatively regulate Tfh cells. Thus, there is a greater deletion of Tfr cells in Bcl6FC mice, leading to a more complete loss of repression by Tfr cells, and thus increased Tfh cell activity. The increased Tfh cell cytokines in Bcl6FC mice might promote the elevated IgA response that is not seen in Stat3FC mice. In summary, in the Bcl6FC model, Tfr cells repress Tfh cell activity but not proliferation. Why Ag-specific IgG is increased in Stat3FC mice is unclear, but possibly the residual Tfr cells in Stat3FC mice have augmented GC helper activity.

T follicular regulatory cells have been studied extensively in immune responses induced by model protein Ags and adjuvants. Studies on Tfr cell function in regulating the gut microbiota (86) or in viral infection (17–19) have also been performed. More than the type of immune challenge, the model system used to assess Tfr cell function (e.g., Treg depletion in mice versus Bcl6FC mice) determines whether suppression by Tfr cells is observed.

ROLE OF Tfr CELLS IN AUTOIMMUNE DISEASE

An important area where Tfr cells have a clear suppressive effect on the GC and Ab response, even in Bcl6FC mice, is in suppression of auto-Abs that drive autoimmune disease (14, 18, 19, 32, 40). This role of Tfr cells in suppressing auto-Ab production was elucidated most thoroughly by Fu et al. who showed that Bcl6FC mice developed late-onset Sjogren's-like autoimmune disease and autoimmunity could be induced in young mice by immunizing mice with salivary gland extracts (18). The precise mechanisms for how Tfr cells can suppress auto-Abs while at the same time promote the Ab response to foreign Ags remains unexplored. One possible explanation is that since Tfr cells, like Tregs, have a bias toward self-Ag recognition (22, 23, 38), they

are able to inhibit self-reactive Tfh cells that might develop in the GC by competing with them for recognition of self-Ags on GC B cells and binding and blocking B7 co-stimulatory receptors *via* CTLA-4. Little is known about the role of Tfr cells in human autoimmune disease, but increased levels of cTfr cells are observed in patients with Sjogren's disease (28, 59) and systemic lupus erythematosus (60). Interestingly, an increased ratio of cTfr to cTfh cells is strongly associated with more severe disease in the case of Sjogren's syndrome (59). Whether high levels of cTfr cells simply represent the presence of active GC responses or whether cTfr cells are especially elevated in autoimmune disease is not clear. The data with Sjogren's cTfr cells is particularly hard to interpret since the cells have an immature CD25⁺ Tfr phenotype and their relationship to GC-localized Tfr cells is unclear (59).

“HELPER” FUNCTIONS OF Tfr CELLS

Although Tregs themselves are overwhelmingly described as suppressor cells, there are several reports that Tregs can promote immune responses in certain circumstances. Under inflammatory conditions or in mice with mutations in genes that affect Foxp3 expression, a fraction of Tregs can become “ex-Tregs” and differentiate into proinflammatory cells (87, 88). Surprisingly, ex-Tregs can also convert into functional Tfh cells in Peyer's patches (89) and in atherosclerosis (90).

The first published characterization of Tfr cells in 2009 by Linterman et al. showed that Tfr cells had a key “helper” role in terms of helping Tfh cells select high-affinity Ag-specific B cell clones (7). In their proposed model, Tfr cells restrict the outgrowth of non-Ag-specific B cell clones in the GC, presumably allowing for more efficient interaction of Tfh cells with selection of specific high-affinity Abs (7). At the same time, the Linterman et al. data can also be interpreted as showing evidence for a major helper function for Tfr cells in the GC. For instance, a significant decrease in Ag-specific GC B cells is observed after total Treg depletion at the same time that total GC B cells increased (7). This can be interpreted as two distinct processes: (1) loss of Tfr cells leads to a loss of Tfr cell helper activity and thus reduced Ag-specific GC B cells and (2) because total Tregs are depleted, there is a massive increase in GC responses to commensals and self-Ags—responses that are normally inhibited by Tregs. Even though these latter commensal-specific and self-Ag-specific GCs may be weakened by loss of Tfr cell helper activity, the large number of these responses leads to a total increase in GC B cells.

A different Treg–Tfh helper pathway was shown by Leon et al., who found that Tregs are required for the normal anti-influenza Tfh cell response (66). In this study, ex-Tregs were not converting into Tfh cells, and Leon et al. proposed a mechanism where CD25⁺ Tregs take up IL-2 and limit the overall availability of IL-2, thereby promoting Tfh cell differentiation (66). Importantly, however, Leon et al. did not investigate loss of Tfr cells (which would occur with Treg depletion) as a mechanism for the Treg helper effect, and their data does not eliminate a helper role for Tfr cells in the Tfh/GC response in the virus infection system.

Because of the problems associated with deleting total Tregs and the lack of specific and robust models to deplete Tfr cells

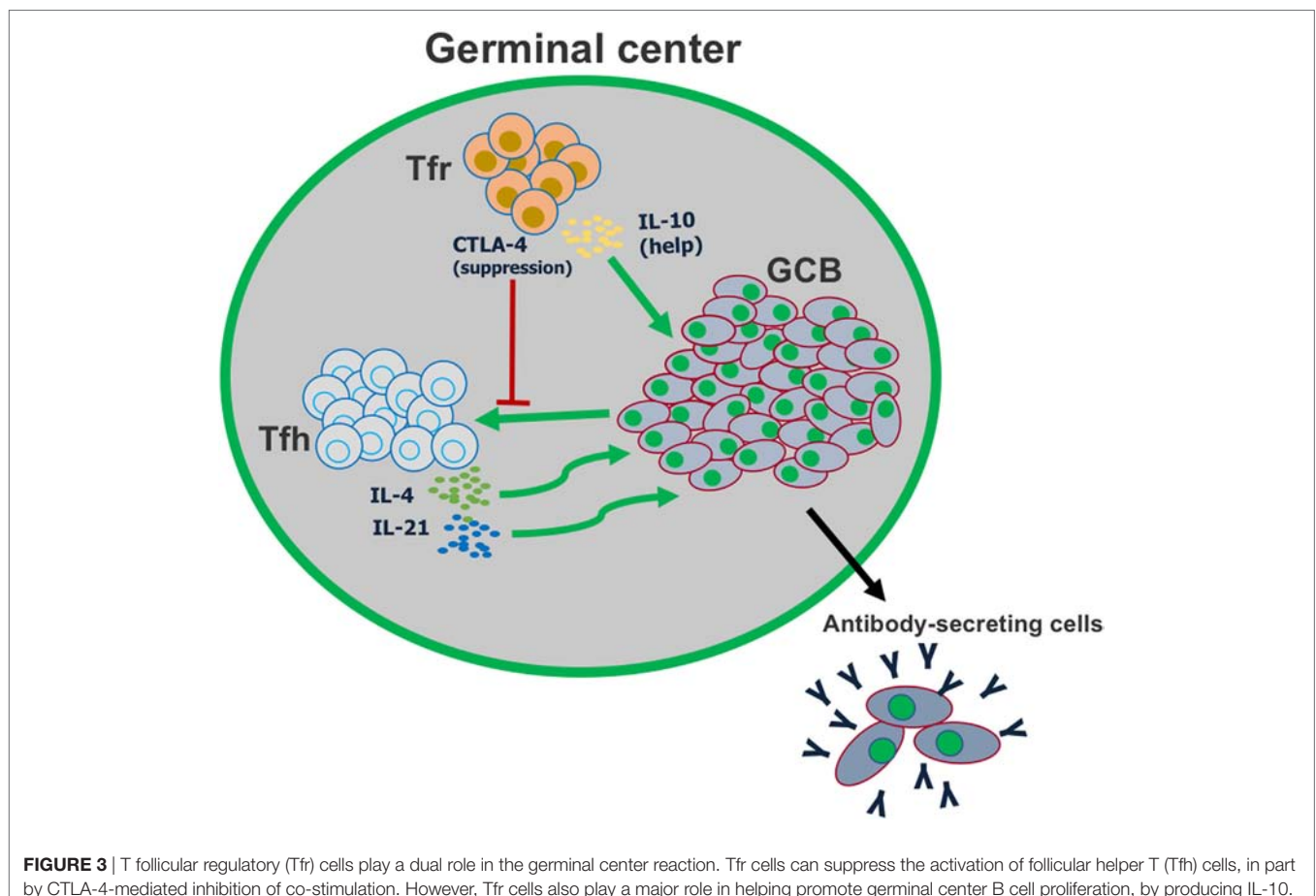
in vivo, we developed Bcl6FC mice (14). In these mice, Tfr cell development is specifically blocked without a loss in total Tregs or Treg function (14). We determined that loss of Tfr cells led to a significantly decreased IgG response and that Tfr cells were required to produce the highest affinity Ag-specific Abs (14). These results are consistent with a critical helper role for Tfr cells in the GC. In our published results, we did not observe a loss of GC B cells or Tfh cells in Bcl6FC mice despite the decreased IgG response (14). This could be due to the time-point where we analyzed the GC or the type of Ag used to induce the GC.

In 2017, Laidlaw et al. presented the clearest evidence to date that Tfr cells can act as essential helper cells in the GC (17). In this study, mice were infected with lymphocytic choriomeningitis virus (LCMV) and the GC and Ab response analyzed (17). Importantly, Laidlaw et al. used Bcl6FC mice and Treg-specific IL-10 cKO mice to demonstrate that Tfr cells are a critical source of IL-10 in the GC and that IL-10 drives the growth of GCs by promoting entry of GC B cells into the dark zone (17). In the absence of IL-10-producing Tfr cells, GC B cell numbers and the LCMV-specific Ab response were decreased (17). A recent study with malaria infection in mice also showed that IL-10 was critical for the maintenance of the GC and GC-derived Ab response (55). Overall, these recent findings strongly support the idea that IL-10-producing Tfr cells have a major role in maintaining the GC reaction and thus act as “helper cells.” In our lab, we have

been using Bcl6FC mice and analyzing the role of Tfr cells in a food allergy model with peanut Ag. In this model, we find that Tfr cells help maintain the peanut-specific GC response and IgE response (Markus M. Xie and Alexander L. Dent, manuscript in preparation). Tfr cells thus appear to have a key role in allergic immune responses, and represent a novel target for allergy-specific immunotherapy.

OUTSTANDING QUESTIONS AND FUTURE DIRECTIONS

As of this writing, Tfr cells have only been analyzed in a very small fraction of infectious disease models and immunological diseases such as allergy and autoimmunity. Testing Tfr cell function in various disease states will be an important area for future research on Tfr cells. Also unknown is if Tfr cells affect diseases that are not driven by Ab-mediated pathology. Whether Tfr cells play a regulatory role in cancer, diabetes, heart disease, atherosclerosis, or other types of inflammatory diseases, is ripe for exploration. The mechanism of Tfr cell help in the GC is not completely understood and an important topic is why some types of GC responses seem to rely on Tfr cells for help whereas other GC responses are only mildly affected or not affected at all by loss of Tfr cells. A major issue for future studies is whether Tfr cells switch between help and suppression in the GC for foreign Ag, or primarily



act as helpers for foreign Ag and suppressors of autoimmune responses. If Tfr cells act as suppressor cells of non-autoimmune responses in the GC, what mechanism of suppression do they use, and what controls whether Tfr cells act as suppressors versus helpers? Do human and mouse Tfr cells have similar helper and repressor functions? Tfr-like cells have been found circulating in both mouse and human; what is the relationship of these cells to Tfr cells in the GC? Also unclear is how Tfr cells regulate Ab affinity maturation and Tfh responses. A major question is whether Tfr cells regulate the generation or differentiation or survival of memory B cells. Finally, almost nothing is known about what signals drive Tfr cell responses to the GC and what Ags do they recognize? Thus, there are huge numbers of vital questions about Tfr cells that need to be answered through more research.

CONCLUSION

Even though Tregs act overwhelmingly as major suppressors of the immune response, Tfr cells provide a striking and clear example of Tregs acting as “helper” cells for the immune response. At least part of this Tfr cell helper function is producing IL-10 that promotes GC B cell growth and the GC-dependent high-affinity

Ab response. Thus, in the context of the GC response, Tfr cells appear to maintain a key balance between help (GC maintenance, Ab response, and Ab affinity) and suppression (Tfh cell numbers, GC B cell numbers, Tfh cell cytokines, and auto-Abs) (**Figure 3**). One interesting idea is that the autoreactivity and suppressive capability of Tregs is used to help control autoimmunity in the GC but has been co-opted to also promote the overall GC response. Much work remains to fully understand the role of Tfr cells in the overall humoral immune response, and in the larger scope of the immune system.

AUTHOR CONTRIBUTIONS

AD and MX both wrote and made equal contributions to the manuscript.

ACKNOWLEDGMENTS

This work was supported by National Institutes of Health R01 AI132771-01 to AD. MX was supported by a Careers in Immunology Fellowship from American Association of Immunologists. We thank Dr. Mark Kaplan for critically reviewing the manuscript.

REFERENCES

- Fazilleau N, Mark L, McHeyzer-Williams LJ, McHeyzer-Williams MG. Follicular helper T cells: lineage and location. *Immunity* (2009) 30:324–35. doi:10.1016/j.immuni.2009.03.003
- Crotty S, Johnston RJ, Schoenberger SP. Effectors and memories: Bcl-6 and Blimp-1 in T and B lymphocyte differentiation. *Nat Immunol* (2010) 11:114–20. doi:10.1038/ni.1837
- McHeyzer-Williams LJ, Pelletier N, Mark L, Fazilleau N, McHeyzer-Williams MG. Follicular helper T cells as cognate regulators of B cell immunity. *Curr Opin Immunol* (2009) 21:266–73. doi:10.1016/j.coi.2009.05.010
- Breitfeld D, Ohl L, Kremmer E, Ellwart J, Sallusto F, Lipp M, et al. Follicular B helper T cells express CXC chemokine receptor 5, localize to B cell follicles, and support immunoglobulin production. *J Exp Med* (2000) 192:1545–52. doi:10.1084/jem.192.11.1545
- Schaerli P, Willmann K, Lang AB, Lipp M, Loetscher P, Moser B. CXC chemokine receptor 5 expression defines follicular homing T cells with B cell helper function. *J Exp Med* (2000) 192:1553–62. doi:10.1084/jem.192.11.1553
- Vinuesa CG, Cook MC, Angelucci C, Athanasopoulos V, Rui L, Hill KM, et al. A RING-type ubiquitin ligase family member required to repress follicular helper T cells and autoimmunity. *Nature* (2005) 435:452–8. doi:10.1038/nature03555
- Linterman MA, Rigby RJ, Wong RK, Yu D, Brink R, Cannons JL, et al. Follicular helper T cells are required for systemic autoimmunity. *J Exp Med* (2009) 206:561–76. doi:10.1084/jem.20081886
- Odegar JM, Marks BR, DiPlacido LD, Poholek AC, Kono DH, Dong C, et al. ICOS-dependent extrafollicular helper T cells elicit IgG production via IL-21 in systemic autoimmunity. *J Exp Med* (2008) 205:2873–86. doi:10.1084/jem.20080840
- Wollenberg I, Agua-Doce A, Hernandez A, Almeida C, Oliveira VG, Faro J, et al. Regulation of the germinal center reaction by Foxp3+ follicular regulatory T cells. *J Immunol* (2011) 187:4553–60. doi:10.4049/jimmunol.1101328
- Linterman MA, Pierson W, Lee SK, Kallies A, Kawamoto S, Rayner TF, et al. Foxp3+ follicular regulatory T cells control the germinal center response. *Nat Med* (2011) 17:975–82. doi:10.1038/nm.2425
- Chung Y, Tanaka S, Chu F, Nurieva RI, Martinez GJ, Rawal S, et al. Follicular regulatory T cells expressing Foxp3 and Bcl-6 suppress germinal center reactions. *Nat Med* (2011) 17:983–8. doi:10.1038/nm.2426
- Sage PT, Alvarez D, Godec J, von Andrian UH, Sharpe AH. Circulating T follicular regulatory and helper cells have memory-like properties. *J Clin Invest* (2014) 124:5191–204. doi:10.1172/JCI76861
- Sage PT, Paterson AM, Lovitch SB, Sharpe AH. The coinhibitory receptor CTLA-4 controls B cell responses by modulating T follicular helper, T follicular regulatory, and T regulatory cells. *Immunity* (2014) 41:1026–39. doi:10.1016/j.immuni.2014.12.005
- Wu H, Chen Y, Liu H, Xu LL, Teuscher P, Wang S, et al. Follicular regulatory T cells repress cytokine production by follicular helper T cells and optimize IgG responses in mice. *Eur J Immunol* (2016) 46:1152–61. doi:10.1002/eji.201546094
- Wu H, Xie MM, Liu H, Dent AL. Stat3 is important for follicular regulatory T cell differentiation. *PLoS One* (2016) 11:e0155040. doi:10.1371/journal.pone.0155040
- Sage PT, Sharpe AH. T follicular regulatory cells. *Immunol Rev* (2016) 271:246–59. doi:10.1111/imr.12411
- Laidlaw BJ, Lu Y, Amezcua RA, Weinstein JS, Vander Heiden JA, Gupta NT, et al. Interleukin-10 from CD4(+) follicular regulatory T cells promotes the germinal center response. *Sci Immunol* (2017) 2:1–10. doi:10.1126/sciimmunol.aan4767
- Fu W, Liu X, Lin X, Feng H, Sun L, Li S, et al. Deficiency in T follicular regulatory cells promotes autoimmunity. *J Exp Med* (2018) 215(3):815–25. doi:10.1084/jem.20170901
- Botta D, Fuller MJ, Marquez-Lago TT, Bachus H, Bradley JE, Weinmann AS, et al. Dynamic regulation of T follicular regulatory cell responses by interleukin 2 during influenza infection. *Nat Immunol* (2017) 18:1249–60. doi:10.1038/ni.3837
- Crotty S. Follicular helper CD4 T cells (TFH). *Annu Rev Immunol* (2011) 29:621–63. doi:10.1146/annurev-immunol-031210-101400
- Crotty S. T follicular helper cell differentiation, function, and roles in disease. *Immunity* (2014) 41:529–42. doi:10.1016/j.immuni.2014.10.004
- Maceiras AR, Almeida SCP, Mariotti-Ferrandiz E, Chaara W, Jebbawi F, Six A, et al. T follicular helper and T follicular regulatory cells have different TCR specificity. *Nat Commun* (2017) 8:15067. doi:10.1038/ncomms15067
- Aloulou M, Carr EJ, Gador M, Bignon A, Liblau RS, Fazilleau N, et al. Follicular regulatory T cells can be specific for the immunizing antigen and derive from naive T cells. *Nat Commun* (2016) 7:10579. doi:10.1038/ncomms10579
- Bilate AM, Lafaille JJ. Induced CD4+Foxp3+ regulatory T cells in immune tolerance. *Annu Rev Immunol* (2012) 30:733–58. doi:10.1146/annurev-immunol-020711-075043

25. Yadav M, Stephan S, Bluestone JA. Peripherally induced Tregs – role in immune homeostasis and autoimmunity. *Front Immunol* (2013) 4:232. doi:10.3389/fimmu.2013.00232
26. Georgiev H, Ravens I, Papadogianni G, Halle S, Malissen B, Loots GG, et al. Shared and unique features distinguishing follicular T helper and regulatory cells of peripheral lymph node and Peyer's patches. *Front Immunol* (2018) 9:714. doi:10.3389/fimmu.2018.00714
27. Sage PT, Francisco LM, Carman CV, Sharpe AH. The receptor PD-1 controls follicular regulatory T cells in the lymph nodes and blood. *Nat Immunol* (2013) 14:152–61. doi:10.1038/ni.2496
28. Fonseca VR, Agua-Doce A, Maceiras AR, Pierson W, Ribeiro F, Romao VC, et al. Human blood Tfr cells are indicators of ongoing humoral activity not fully licensed with suppressive function. *Sci Immunol* (2017) 2:1–12. doi:10.1126/sciimmunol.aan1487
29. Wing JB, Kitagawa Y, Locci M, Hume H, Tay C, Morita T, et al. A distinct subpopulation of CD25(-) T-follicular regulatory cells localizes in the germinal centers. *Proc Natl Acad Sci U S A* (2017) 114:E6400–9. doi:10.1073/pnas.1705551114
30. Vaccari M, Franchini G. T cell subsets in the germinal center: lessons from the macaque model. *Front Immunol* (2018) 9:348. doi:10.3389/fimmu.2018.00348
31. Xie MM, Koh BH, Hollister K, Wu H, Sun J, Kaplan MH, et al. Bcl6 promotes follicular helper T-cell differentiation and PD-1 expression in a Blimp1-independent manner in mice. *Eur J Immunol* (2017) 47:1136–41. doi:10.1002/eji.201747034
32. Vaeth M, Muller G, Stauss D, Dietz L, Klein-Hessling S, Serfling E, et al. Follicular regulatory T cells control humoral autoimmunity via NFAT2-regulated CXCR5 expression. *J Exp Med* (2014) 211:545–61. doi:10.1084/jem.20130604
33. Xu L, Huang Q, Wang H, Hao Y, Bai Q, Hu J, et al. The kinase mTORC1 promotes the generation and suppressive function of follicular regulatory T cells. *Immunity* (2017) 47:538–51.e5. doi:10.1016/j.immuni.2017.08.011
34. Essig K, Hu D, Guimaraes JC, Alterauge D, Edelmann S, Raj T, et al. Roquin suppresses the PI3K-mTOR signaling pathway to inhibit T helper cell differentiation and conversion of Treg to Tfr cells. *Immunity* (2017) 47:1067–82.e12. doi:10.1016/j.immuni.2017.11.008
35. Shrestha S, Yang K, Guy C, Vogel P, Neale G, Chi H. Treg cells require the phosphatase PTEN to restrain TH1 and TFH cell responses. *Nat Immunol* (2015) 16:178–87. doi:10.1038/ni.3076
36. Sage PT, Schildberg FA, Sobel RA, Kuchroo VK, Freeman GJ, Sharpe AH. Dendritic cell PD-L1 limits autoimmunity and follicular T cell differentiation and function. *J Immunol* (2018) 200(8):2592–602. doi:10.4049/jimmunol.1701231
37. Chruscinski A, Sadozai H, Rojas-Luengas V, Bartczak A, Khattar R, Selzner N, et al. Role of regulatory T cells (Treg) and the Treg effector molecule fibrinogen-like protein 2 in alloimmunity and autoimmunity. *Rambam Maimonides Med J* (2015) 6:1–17. doi:10.5041/RMMJ.10209
38. Ritvo PG, Churlaud G, Quiniou V, Florez L, Brimard F, Fourcade G, et al. Tfr cells lack IL-2Ralpha but express decoy IL-1R2 and IL-1Ra and suppress the IL-1-dependent activation of Tfh cells. *Sci Immunol* (2017) 2:1–11. doi:10.1126/sciimmunol.aan0368
39. Gopisetty A, Bhattacharya P, Haddad C, Bruno JC Jr, Vasu C, Miele L, et al. OX40L/Jagged1 cosignaling by GM-CSF-induced bone marrow-derived dendritic cells is required for the expansion of functional regulatory T cells. *J Immunol* (2013) 190:5516–25. doi:10.4049/jimmunol.1202298
40. Zhang R, Sage PT, Finn K, Huynh A, Blazar BR, Marangoni F, et al. B cells drive autoimmunity in mice with CD28-deficient regulatory T cells. *J Immunol* (2017) 199:3972–80. doi:10.4049/jimmunol.171.7.3348
41. Linterman MA, Denton AE, Divekar DP, Zvetkova I, Kane L, Ferreira C, et al. CD28 expression is required after T cell priming for helper T cell responses and protective immunity to infection. *Elife* (2014) 3:1–21. doi:10.7554/eLife.03180
42. Franckaert D, Dooley J, Roos E, Floess S, Huehn J, Luche H, et al. Promiscuous Foxp3-cre activity reveals a differential requirement for CD28 in Foxp3(+) and Foxp3(-) T cells. *Immunol Cell Biol* (2015) 93:417–23. doi:10.1038/icb.2014.108
43. Tang Q, Henriksen KJ, Boden EK, Tooley AJ, Ye J, Subudhi SK, et al. Cutting edge: CD28 controls peripheral homeostasis of CD4+CD25+ regulatory T cells. *J Immunol* (2003) 171:3348–52. doi:10.4049/jimmunol.171.7.3348
44. Tai X, Cowan M, Feigenbaum L, Singer A. CD28 costimulation of developing thymocytes induces Foxp3 expression and regulatory T cell differentiation independently of interleukin 2. *Nat Immunol* (2005) 6:152–62. doi:10.1038/ni1160
45. Choi YS, Kageyama R, Eto D, Escobar TC, Johnston RJ, Monticelli L, et al. ICOS receptor instructs T follicular helper cell versus effector cell differentiation via induction of the transcriptional repressor Bcl6. *Immunity* (2011) 34:932–46. doi:10.1016/j.immuni.2011.03.023
46. Xu H, Li X, Liu D, Li J, Zhang X, Chen X, et al. Follicular T-helper cell recruitment governed by bystander B cells and ICOS-driven motility. *Nature* (2013) 496:523–7. doi:10.1038/nature12058
47. Bauquet AT, Jin H, Paterson AM, Mitsdoerffer M I, Ho C, Sharpe AH, et al. The costimulatory molecule ICOS regulates the expression of c-Maf and IL-21 in the development of follicular T helper cells and TH-17 cells. *Nat Immunol* (2009) 10:167–75. doi:10.1038/ni.1690
48. Wheaton JD, Yeh CH, Ciofani M. Cutting edge: c-Maf is required for regulatory T cells To adopt RORgammat(+) and follicular phenotypes. *J Immunol* (2017) 199:3931–6. doi:10.4049/jimmunol.1701134
49. Xu M, Pokrovskii M, Ding Y, Yi R, Au C, Harrison OJ, et al. c-MAF-dependent regulatory T cells mediate immunological tolerance to a gut pathobiont. *Nature* (2018) 554:373–7. doi:10.1038/nature25500
50. Johnston RJ, Poholek AC, DiToro D, Yusuf I, Eto D, Barnett B, et al. Bcl6 and Blimp-1 are reciprocal and antagonistic regulators of T follicular helper cell differentiation. *Science* (2009) 325:1006–10. doi:10.1126/science.1175870
51. Lu P, Youngblood BA, Austin JW, Mohammed AU, Butler R, Ahmed R, et al. Blimp-1 represses CD8 T cell expression of PD-1 using a feed-forward transcriptional circuit during acute viral infection. *J Exp Med* (2014) 211:515–27. doi:10.1084/jem.20130208
52. Alegre ML, Frauwirth KA, Thompson CB. T-cell regulation by CD28 and CTLA-4. *Nat Rev Immunol* (2001) 1:220–8. doi:10.1038/35105024
53. Wing JB, Ise W, Kurosaki T, Sakaguchi S. Regulatory T cells control antigen-specific expansion of Tfh cell number and humoral immune responses via the coreceptor CTLA-4. *Immunity* (2014) 41:1013–25. doi:10.1016/j.immuni.2014.12.006
54. Paterson AM, Lovitch SB, Sage PT, Juneja VR, Lee Y, Trombley JD, et al. Deletion of CTLA-4 on regulatory T cells during adulthood leads to resistance to autoimmunity. *J Exp Med* (2015) 212:1603–21. doi:10.1084/jem.20141030
55. Guthmiller JJ, Graham AC, Zander RA, Pope RL, Butler NS. Cutting edge: IL-10 is essential for the generation of germinal center B cell responses and anti-plasmodium humoral immunity. *J Immunol* (2017) 198:617–22. doi:10.4049/jimmunol.1601762
56. Sayin I, Radtke AJ, Vella LA, Jin W, Wherry EJ, Buggert M, et al. Spatial distribution and function of T follicular regulatory cells in human lymph nodes. *J Exp Med* (2018) 215:1531–42. doi:10.1084/jem.20171940
57. Wallin EF, Jolly EC, Suchanek O, Bradley JA, Espeli M, Jayne DR, et al. Human T-follicular helper and T-follicular regulatory cell maintenance is independent of germinal centers. *Blood* (2014) 124:2666–74. doi:10.1182/blood-2014-07-585976
58. Ma CS, Wong N, Rao G, Avery DT, Torpy J, Hambridge T, et al. Monogenic mutations differentially affect the quantity and quality of T follicular helper cells in patients with human primary immunodeficiencies. *J Allergy Clin Immunol* (2015) 136:993–1006.e1. doi:10.1016/j.jaci.2015.05.036
59. Fonseca VR, Romao VC, Agua-Doce A, Santos M, Lopez-Presa D, Ferreira AC, et al. The ratio of blood T follicular regulatory cells to T follicular helper cells marks ectopic lymphoid structure formation while activated follicular helper T cells indicate disease activity in primary Sjogren's syndrome. *Arthritis Rheumatol* (2018) 70:774–84. doi:10.1002/art.40424
60. Liu C, Wang D, Song Y, Lu S, Zhao J, Wang H. Increased circulating CD4(+) CXCR5(+)FoxP3(+) follicular regulatory T cells correlated with severity of systemic lupus erythematosus patients. *Int Immunopharmacol* (2018) 56:261–8. doi:10.1016/j.intimp.2018.01.038
61. Shan Y, Qi C, Zhao J, Liu Y, Gao H, Zhao D, et al. Higher frequency of peripheral blood follicular regulatory T cells in patients with new onset ankylosing spondylitis. *Clin Exp Pharmacol Physiol* (2015) 42:154–61. doi:10.1111/1440-1681.12330
62. Charbonnier LM, Janssen E, Chou J, Ohsumi TK, Keles S, Hsu JT, et al. Regulatory T-cell deficiency and immune dysregulation, polyendocrinopathy, enteropathy, X-linked-like disorder caused by loss-of-function mutations in LRBA. *J Allergy Clin Immunol* (2015) 135:217–27. doi:10.1016/j.jaci.2014.10.019

63. Kucuk ZY, Charbonnier LM, McMasters RL, Chatila T, Bleesing JJ. CTLA-4 haploinsufficiency in a patient with an autoimmune lymphoproliferative disorder. *J Allergy Clin Immunol* (2017) 140:862–4.e4. doi:10.1016/j.jaci.2017.02.032
64. Alroqi FJ, Charbonnier LM, Baris S, Kiykim A, Chou J, Platt CD, et al. Exaggerated follicular helper T-cell responses in patients with LRBA deficiency caused by failure of CTLA4-mediated regulation. *J Allergy Clin Immunol* (2018) 141:1050–9.e10. doi:10.1016/j.jaci.2017.05.022
65. Sage PT, Ron-Harel N, Juneja VR, Sen DR, Maleri S, Sungnak W, et al. Suppression by TFR cells leads to durable and selective inhibition of B cell effector function. *Nat Immunol* (2016) 17:1436–46. doi:10.1038/ni.3578
66. Leon B, Bradley JE, Lund FE, Randall TD, Ballesteros-Tato A. FoxP3+ regulatory T cells promote influenza-specific Tfh responses by controlling IL-2 availability. *Nat Commun* (2014) 5:3495. doi:10.1038/ncomms4495
67. Tang Q, Bluestone JA. The Foxp3+ regulatory T cell: a jack of all trades, master of regulation. *Nat Immunol* (2008) 9:239–44. doi:10.1038/ni1572
68. Vignali DA, Collison LW, Workman CJ. How regulatory T cells work. *Nat Rev Immunol* (2008) 8:523–32. doi:10.1038/nri2343
69. Pillai MR, Collison LW, Wang X, Finkelstein D, Rehman JE, Boyd K, et al. The plasticity of regulatory T cell function. *J Immunol* (2011) 187:4987–97. doi:10.4049/jimmunol.1102173
70. Nurieva RI, Podd A, Chen Y, Alekseev AM, Yu M, Qi X, et al. STAT5 protein negatively regulates T follicular helper (Tfh) cell generation and function. *J Biol Chem* (2012) 287:11234–9. doi:10.1074/jbc.M111.324046
71. Johnston RJ, Choi YS, Diamond JA, Yang JA, Crotty S. STAT5 is a potent negative regulator of TFH cell differentiation. *J Exp Med* (2012) 209:243–50. doi:10.1084/jem.20111174
72. Ballesteros-Tato A, Leon B, Graf BA, Moquin A, Adams PS, Lund FE, et al. Interleukin-2 inhibits germinal center formation by limiting T follicular helper cell differentiation. *Immunity* (2012) 36:847–56. doi:10.1016/j.immuni.2012.02.012
73. Collison LW, Delgoffe GM, Guy CS, Vignali KM, Chaturvedi V, Fairweather D, et al. The composition and signaling of the IL-35 receptor are unconventional. *Nat Immunol* (2012) 13:290–9. doi:10.1038/ni.2227
74. Marshall HD, Ray JP, Laidlaw BJ, Zhang N, Gawande D, Staron MM, et al. The transforming growth factor beta signaling pathway is critical for the formation of CD4 T follicular helper cells and isotype-switched antibody responses in the lung mucosa. *Elife* (2015) 4:e04851. doi:10.7554/eLife.04851
75. McCarron MJ, Marie JC. TGF-beta prevents T follicular helper cell accumulation and B cell autoreactivity. *J Clin Invest* (2014) 124:4375–86. doi:10.1172/JCI76179
76. Schmitt N, Liu Y, Bentebibel SE, Munagala I, Bourdery L, Venuprasad K, et al. The cytokine TGF-beta co-opts signaling via STAT3-STAT4 to promote the differentiation of human TFH cells. *Nat Immunol* (2014) 15:856–65. doi:10.1038/ni.2947
77. Travert M, Ame-Thomas P, Pangault C, Morizot A, Mischeau O, Semana G, et al. CD40 ligand protects from TRAIL-induced apoptosis in follicular lymphomas through NF-kappaB activation and up-regulation of c-FLIP and Bcl-xL. *J Immunol* (2008) 181:1001–11. doi:10.4049/jimmunol.181.2.1001
78. Joller N, Lozano E, Burkett PR, Patel B, Xiao S, Zhu C, et al. Treg cells expressing the coinhibitory molecule TIGIT selectively inhibit proinflammatory Th1 and Th17 cell responses. *Immunity* (2014) 40:569–81. doi:10.1016/j.immuni.2014.02.012
79. Liu H, Shalev I, Manuel J, He W, Leung E, Crookshank J, et al. The FGL2-FcgammaRIIB pathway: a novel mechanism leading to immunosuppression. *Eur J Immunol* (2008) 38:3114–26. doi:10.1002/eji.200838338
80. Bolland S, Yim YS, Tus K, Wakeland EK, Ravetch JV. Genetic modifiers of systemic lupus erythematosus in FcgammaRIIB(-/-) mice. *J Exp Med* (2002) 195:1167–74. doi:10.1084/jem.20020165
81. Shalev I, Liu H, Kosciak C, Bartczak A, Javadi M, Wong KM, et al. Targeted deletion of fgl2 leads to impaired regulatory T cell activity and development of autoimmune glomerulonephritis. *J Immunol* (2008) 180:249–60. doi:10.4049/jimmunol.180.1.249
82. Mestecky J, Novak J, Moldoveanu Z, Raska M. IgA nephropathy enigma. *Clin Immunol* (2016) 172:72–7. doi:10.1016/j.clim.2016.07.011
83. Yu X, Harden K, Gonzalez LC, Francesco M, Chiang E, Irving B, et al. The surface protein TIGIT suppresses T cell activation by promoting the generation of mature immunoregulatory dendritic cells. *Nat Immunol* (2009) 10:48–57. doi:10.1038/ni.1674
84. Nurieva RI, Chung Y, Hwang D, Yang XO, Kang HS, Ma L, et al. Generation of T follicular helper cells is mediated by interleukin-21 but independent of T helper 1, 2, or 17 cell lineages. *Immunity* (2008) 29:138–49. doi:10.1016/j.immuni.2008.05.009
85. Poholek AC, Hansen K, Hernandez SG, Eto D, Chandele A, Weinstein JS, et al. In vivo regulation of Bcl6 and T follicular helper cell development. *J Immunol* (2010) 185:313–26. doi:10.4049/jimmunol.0904023
86. Kawamoto S, Maruya M, Kato LM, Suda W, Atarashi K, Doi Y, et al. Foxp3(+) T cells regulate immunoglobulin a selection and facilitate diversification of bacterial species responsible for immune homeostasis. *Immunity* (2014) 41:152–65. doi:10.1016/j.immuni.2014.05.016
87. Sawant DV, Wu H, Yao W, Sehra S, Kaplan MH, Dent AL. The transcriptional repressor Bcl6 controls the stability of regulatory T cells by intrinsic and extrinsic pathways. *Immunology* (2015) 145:11–23. doi:10.1111/imm.12393
88. Sawant DV, Vignali DA. Once a Treg, always a Treg? *Immunol Rev* (2014) 259:173–91. doi:10.1111/imr.12173
89. Tsuji M, Komatsu N, Kawamoto S, Suzuki K, Kanagawa O, Honjo T, et al. Preferential generation of follicular B helper T cells from Foxp3+ T cells in gut Peyer's patches. *Science* (2009) 323:1488–92. doi:10.1126/science.1169152
90. Gaddis DE, Padgett LE, Wu R, McSkimming C, Romines V, Taylor AM, et al. Apolipoprotein AI prevents regulatory to follicular helper T cell switching during atherosclerosis. *Nat Commun* (2018) 9:1095. doi:10.1038/s41467-018-03493-5

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2018 Xie and Dent. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



T Follicular Helper Cells in Autoimmune Disorders

Noémie Gensous, Manon Charrier, Dorothée Duluc, Cécile Contin-Bordes, Marie-Elise Truchetet, Estibaliz Lazaro, Pierre Duffau, Patrick Blanco and Christophe Richez*

ImmunoConcept, UMR-CNRS 5164, Université de Bordeaux, Bordeaux, France

T follicular helper (Tfh) cells are a distinct subset of CD4⁺ T lymphocytes, specialized in B cell help and in regulation of antibody responses. They are required for the generation of germinal center reactions, where selection of high affinity antibody producing B cells and development of memory B cells occur. Owing to the fundamental role of Tfh cells in adaptive immunity, the stringent control of their production and function is critically important, both for the induction of an optimal humoral response against thymus-dependent antigens but also for the prevention of self-reactivity. Indeed, deregulation of Tfh activities can contribute to a pathogenic autoantibody production and can play an important role in the promotion of autoimmune diseases. In the present review, we briefly introduce the molecular factors involved in Tfh cell formation in the context of a normal immune response, as well as markers associated with their identification (transcription factor, surface marker expression, and cytokine production). We then consider in detail the role of Tfh cells in the pathogenesis of a broad range of autoimmune diseases, with a special focus on systemic lupus erythematosus and rheumatoid arthritis, as well as on the other autoimmune/inflammatory disorders. We summarize the observed alterations in Tfh numbers, activation state, and circulating subset distribution during autoimmune and some other inflammatory disorders. In addition, central role of interleukin-21, major cytokine produced by Tfh cells, is discussed, as well as the involvement of follicular regulatory T cells, which share characteristics with both Tfh and regulatory T cells.

Keywords: auto-immunity, auto-immune diseases, T cells, T helper follicular cells, systemic lupus erythematosus

OPEN ACCESS

Edited by:

Georgia Foustari,
San Raffaele Hospital (IRCCS), Italy

Reviewed by:

Tri Giang Phan,
Garvan Institute of Medical
Research, Australia
Laurence Morel,
University of Florida, United States

*Correspondence:

Christophe Richez
christophe.richez@chu-bordeaux.fr

Specialty section:

This article was submitted
to T Cell Biology,
a section of the journal
Frontiers in Immunology

Received: 05 May 2018

Accepted: 03 July 2018

Published: 17 July 2018

Citation:

Gensous N, Charrier M, Duluc D,
Contin-Bordes C, Truchetet M-E,
Lazaro E, Duffau P, Blanco P and
Richez C (2018) T Follicular Helper
Cells in Autoimmune Disorders.
Front. Immunol. 9:1637.
doi: 10.3389/fimmu.2018.01637

INTRODUCTION

T follicular helper (Tfh) cells represent a distinct CD4⁺ helper T cell subset, specialized for provision of help to B cells (1–4). They develop within secondary lymphoid organs (SLO) and can be identified based on their unique surface phenotype, cytokine secretion profile, and signature transcription factor. They support B cells to produce high-affinity antibodies toward antigens (Ag), in order to develop a robust humoral immune response and they are crucial for the generation of B cell memory. Whereas they are essential for infectious disease control and optimal antibody responses after vaccination, an excessive response can lead to a breakdown of tolerance. In this review, after introducing the biology of Tfh cells, we will consider in detail their role in the pathogenesis of autoimmune diseases and inflammatory disorders.

Tfh CELLS: OVERVIEW ON CHARACTERIZATION, GENERATION, AND FUNCTIONS

T follicular helper cells were initially described in 2000 and 2001 in humans (1–4). They have been identified as a distinct T helper cell subset, based on their unique combination of surface

markers [abundant expression of chemokine (C-X-C motif) receptor 5 (CXCR5) (1, 4), downregulation of C-C chemokine receptor type 7 (CCR7), and expression of the co-stimulatory molecules inducible co-stimulator (ICOS) (1) and programmed cell death protein-1 (PD-1)], cytokine production [expression of high levels of interleukin 21 (IL-21)], and specific transcription factor [expression of the nuclear transcriptional repressor B cell lymphoma 6 (Bcl-6)]. All are necessary for the development, the maintenance, and the functions of these cells. Tfh cells are crucial for the generation of germinal centers (GC) (5, 6), unique structures formed in SLO where antigen-primed B cells undergo proliferation, immunoglobulin (Ig) class switching, somatic hypermutation, and differentiation (7). Strong expression of CXCR5 by Tfh cells is pivotal for their migration into B cell follicles, in which they are attracted in response to the gradient expression of chemokine ligand 13 (CXCL13) (8–10). Within GC, Tfh cells are important regulators of B cell maturation and help signals rely on both cell-to-cell interactions and on the production of cytokines. Inside GC, Tfh cells are equipped with a specific combination of surface molecules, such as ICOS, a co-stimulatory molecule that belongs to the CD28 superfamily, PD-1, a potent inhibitory receptor, or CD40 ligand (CD40L), a member of the tumor necrosis factor family. Engagement of these molecules on their receptors on B cells delivers signals of survival, differentiation, or isotype switching (11–22). Tfh cells are also characterized by the secretion of a diversity of cytokines (23–26). Production of high levels of IL-21 is one hallmark of Tfh cells (23). This cytokine promotes B cells differentiation into plasma cells, somatic hypermutation, and Ig isotype switching (23, 27–30). Moreover, IL-21 promotes Tfh cells differentiation in a positive autocrine feedback loop (31–35).

Differentiation and development of Tfh cells is an extremely complex process, occurring in a multi-stage way. The essential transcription factor of Tfh cells, Bcl-6, controls their differentiation, their full maturation, and the expression of Tfh cells signature molecules (36–38). Bcl-6 expression, associated with the downregulation of its antagonist Blimp-1, leads to the inhibition of the other transcription factors specific to other T helper cell lineages (T-bet, GATA3, and ROR γ t specially). In addition to Bcl-6, other transcription factors, such as c-MAF, achaete-scute complex homolog 2, basic leucine zipper transcription factor (39), or IFN regulatory factor 4 are also crucial for Tfh cells differentiation (39–41). While Bcl-6 expression is essential for Tfh cells, it should be noted that it is not perfectly Tfh cell-specific, as it can be transiently up-regulated in dividing CD4⁺ T cells after activation by dendritic cells (42, 43). Micro-environmental factors are necessary for T cells to acquire a Tfh profile. First, Ag stimulation and constant initial interaction with Ag-presenting cells (APCs) and activated B cells play a crucial role in their differentiation from naive CD4⁺ T cells (2, 44–46). Second, a specific cytokinic milieu is necessary as IL-12, and to a lesser extent TGF β , seem to be of particular importance for Tfh cells differentiation in humans (47–50). In addition, although originally recognized as an essential T-cell growth factor, IL-2 is a potent inhibitor of Tfh cells differentiation as the ligation of its receptor leads to STAT5 activation, promoting the formation of non-Tfh effector cells (51–53).

One layer of complexity in the study of Tfh cell populations is their substantial heterogeneity. Tfh cells are not a monolithic population with fixed phenotype and functions. They undergo changes over time in the expression of surface molecules and in the secretion of cytokines, in order to shape more efficiently the help delivered to B cells (45, 54, 55).

The biology of Tfh cells has been extensively studied in SLO in mouse models, but due to the difficulties of sampling in humans, different research groups have focused on identifying the circulating counterparts of bona fide Tfh cells in peripheral blood. Description of a CXCR5⁺ subset of memory CD4⁺ T cells has emerged few years ago (56–58) and has been subsequently referred to as circulating Tfh (cTfh) cells (59). Although these cells do not express Bcl6, they share phenotypic and functional properties with tissue Tfh cells (15, 56, 58–60). However, several questions are still under investigation regarding this population: their exact biology is still poorly defined, and how they relate to bona fide lymphoid Tfh cells remains unclear. Literature data, arising specially from SAP-deficient mice, suggest that development of cTfh cells does not require GC formation. These cells are committed to the Tfh lineage and are primarily generated before participating in GC reaction (54, 58). There is actually no consensus regarding phenotypic markers that should be used for the identification of this population. It is clear that other activated CD4⁺ T cell subsets, with other effector fates, can express transiently CXCR5, ICOS, or PD-1, but we can consider that sustained and/or high levels of expression of these molecules are characteristic of Tfh cells (59). The heterogeneous population of cTfh cells contains three major subsets, defined by their expression of the chemokine receptors CXCR3 and CCR6. They present similar properties with Th1, Th2, and Th17 cells: CXCR3⁺CCR6[−] represent cTfh1 cells, CXCR3[−]CCR6[−] cTfh2 cells and CXCR3[−]CCR6⁺ cTfh17 cells. cTfh2 and cTfh17 are more efficient than cTfh1 cells in helping B cells to proliferate and differentiate into plasma cells (56). Finally, within each of these subsets (cTfh1, cTfh2, or cTfh17), it is possible to subdivide other distinct subpopulations, according to the differential expression of the markers ICOS and PD-1 which can be used as indicators of active state of Tfh cells (58, 61, 62).

Regulatory pathways need to be engaged to control the development and maturation of Tfh cells, as well as their interactions with B cells. Within the diverse regulating mechanisms that we will not develop here, it is of particular importance to mention the role of another specific subset of CD4⁺Foxp3⁺ regulatory T cells, called follicular regulatory T (Tfr) cells, that has been more recently described. These cells share phenotypic characteristics with Tfh cells, with the expression of Bcl-6, CXCR5, PD-1, and ICOS. They are found within the GC, where they act to limit the size of the response, regulating the frequencies of both Tfh and GC B cells (63–66). Lack of Tfr cells is associated with greater GC reactions *in vivo*.

Tfh CELLS AND AUTO-IMMUNITY: GENERALITIES

Autoimmune diseases are caused by a breakdown of immune tolerance. Proliferation of self-reactive B cells with generation of

high-affinity autoantibodies participate to the pathophysiology of these disorders and, in this way, have led to consider Tfh cells as possible actors in their pathogenesis. Moreover, in autoimmune diseases, inflammatory sites develop frequently lymphoid cell aggregations which include B and T helper cells, leading to the formation of ectopic lymphoid structures (67). These tissue-localized T-B-cell interactions which can promote the maturation of B cells and can potentiate the production of pathogenic autoantibodies (68), even if the exact nature of helper T cells in inflamed tissues is not completely understood currently.

Studies of the contribution of Tfh cells in autoimmune disorders have been initially limited to animal models, essentially because of difficulties in investigating Tfh cells from human SLO. The ulterior identification of circulating Tfh-like cells, which are more accessible than Tfh cells in tissues, has provided an opportunity to analyze these cells in the context of human autoimmune disorders. Since then, alterations in Tfh cells have been described in a broad range of autoimmune diseases, including systemic diseases, or organ- and cell-specific disorders. Increased numbers of cTfh cells, often correlating with the frequency of circulating plasmablasts or plasma cells, have been observed in several auto-immune diseases and alterations have also been reported regarding their polarization, their function, and the quality of help they provide. All the data collected so far in auto-immunity and in inflammatory diseases should not mask our lack of knowledge regarding the exact biology of cTfh cells and that analysis could be confounded by our incomplete understanding. Moreover, it is to mention that combination of markers used to define cTfh cell populations in the studies that will be mentioned in the next sections often differ among the laboratories. Some studies have defined cTfh cells as total CXCR5⁺CD4⁺ T cells, while other have investigated a subset of CXCR5⁺CD4⁺ T cells (CXCR5⁺ICOS⁺, CXCR5⁺PD1⁺, CXCR5⁺ICOS⁺PD1⁺, or CXCR5⁺IL-21⁺).

SYSTEMIC LUPUS ERYTHEMATOSUS (SLE)

Systemic lupus erythematosus is characterized by the presence of high-affinity pathogenic autoantibodies directed against nuclear components (69). Autoreactive B cells in SLE are derived from GC reactions (70); therefore, investigations of Tfh cell roles have been intensive since many years, both in murine models and in human SLE. In different mouse models sharing immunopathologic features with human SLE, it has been demonstrated that Tfh cells contribute to the activation of the immune system and to the pathogenesis of the disease. Animals in these different models have an aberrant expansion of Tfh cells, associated with dysregulation of the GC responses. BXD2 mice, which develop spontaneously autoantibodies and glomerulonephritis, are characterized by an increased frequency of PD1⁺ Tfh cells as compared to wild-type animals, correlating positively with the frequency of GC B cells and with levels of anti-dsDNA antibodies (71). Sanroque mice have a particular single recessive mutation in *roquin*, a gene encoding for a RING-type ubiquitin ligase family member that disrupts a repressor of ICOS expression. Animals are characterized by a SLE-like phenotype with lymphadenopathy and splenomegaly, high levels of anti-nuclear antibodies (AAN),

autoimmune cytopenia, and immune complex deposits (72). Even without antigenic stimulation, a spontaneous formation of GC is observed, with an increase in the number of Tfh cells and an increase in their activity, particularly marked regarding the excessive production of IL-21 (72–75). The other murine model that has provided evidence of the implication of Tfh cells in the development of systemic autoimmunity is represented by BXS mice, in which the duplication of the *Thr7* gene promotes excessive signaling by self-autoantigens, leading to a severe inflammatory disease, with high levels of autoantibodies and proliferative glomerulonephritis (76). These animals have expanded numbers of B cells and Tfh cells, especially in the spleen (77). Besides, IL-21 is elevated in the serum and over-expressed in splenocytes (78). In this mouse model, IL-21R deficiency induces a decrease in the serum levels of AAN and prevents the apparition of renal disease (77). Moreover, therapeutic blockade of IL-21, by the administration of an IL-21R-Fc fusion protein, seems to have a biphasic response characterized by an aggravation of the disease when the treatment is given during early life and an anti-inflammatory effect (decrease in IgG1 serum levels, in proteinuria levels, and in histological glomerulonephritis) when it is administrated later in the disease course (79, 80). Implication of IL-21 in the pathogenesis of SLE is also supported by data obtained in two other murine models of the disease. In MRL-Fas(lpr) mice, accumulation of activated B cells, activated T cells, plasma cells, and spontaneous GC formation is dependent on IL-21R signaling. Administration of IL-21R-Fc fusion protein reduces disease severity (81, 82). Finally, in the NZB/NZW mouse model, blockade of IL-21R inhibits the progression of the pre-established disease (83). Murine models also point out the role of interactions between ICOS and its ligand in the development of systemic autoimmunity and suggest that this pathway could possibly be an interesting novel therapeutic target. In the NZB/NZW mice, blockade of ICOS pathway, by the use of a monoclonal antibody directed against ICOS-L, leads to the inhibition of the development of Tfh cells, to a decrease in anti-dsDNA antibody titers and to an improvement in kidney function (84, 85). Reduction in titers of anti-dsDNA antibodies is also observed in the mouse model MRL-Fas(lpr) when animals have an additional genetic deletion in ICOS (86). Finally, in B6.Sle1 mice, elevated expression of ICOS contributes to the expansion of Tfh cells and to the breakdown in peripheral tolerance (87).

Several lines of evidence also support a pathogenic role of Tfh cells and IL-21 in human SLE (Table 1). Data are available mostly for cTfh cells, which have been showed to be increased and have an active phenotype in SLE patients as compared to controls (88–98). This activated phenotype correlates with the number of circulating plasmablasts and with the levels of pathogenic autoantibodies (58, 88, 90, 91, 93–95, 98), but the correlation with disease activity observed in some studies (58, 90, 92, 97) has been considered inconsistent by other research teams (88, 94). Of particular interest are the observations made on alterations in the composition of cTfh cells subsets in SLE, associated with disease activity (99). Ratio of cTfh2 and cTfh17 (which are both considered as efficient B helpers) over cTfh1 are increased in SLE patients as compared to controls and disease activity correlates with the frequency of cTfh2 cells (99). Higher plasma levels of

TABLE 1 | T follicular helper (Tfh) cells in human SLE.

Study	Patients (n)	
Circulating Tfh		
He et al. (58)	26	Expansion of cTfh cells
Simpson et al. (88)	46	Expansion of cTfh cells
		No association with disease activity. Correlation with the titers of autoantibodies
Terrier et al. (89)	25	Expansion of cTfh cells
Feng et al. (90)	42	Expansion of cTfh cells
		Correlation with disease activity and with the titers of autoantibodies
Wang et al. (91)	23	Expansion of cTfh cells
Yang et al. (92)	30	Expansion of cTfh cells in patients with active SLE
		Correlation with disease activity
Zhang et al. (93)	29	Expansion of cTfh cells
		Correlation with the titers of autoantibodies
Choi et al (94)	57	Expansion of cTfh cells
		Correlation with disease activity and with the titers of autoantibodies
Szabo et al. (95)	25	Expansion of cTfh cells
		Correlation with the titers of autoantibodies
Umeda et al. (96)	64	Expansion of cTfh cells
Xu et al. (97)	40	Expansion of cTfh cells
		Correlation with disease activity and with the titers of autoantibodies
Huang et al. (98)	70	Expansion of cTfh cells
		Correlation with disease activity and with the titers of autoantibodies
Le Coz et al. (99)	23	Expansion of cTfh2 cells in patients with active SLE. Decrease in Tfh1 cell subset percentage
Tissue level		
Liarski et al. (105)	42	Identification of functional Tfh cells in lesions of lupus nephritis. Close proximity between B cells and Tfh cells
Chang et al. (106)	68	Presence of T:B cell aggregates or GCs in the tubulointerstitial infiltrate of lupus nephritis

cTfh cells, circulating Tfh cells; GC, germinal center; SLE, systemic lupus erythematosus.

IL-21, as well as an increase in the frequency of IL-21-expressing CD4⁺ T cells, are found in SLE patients as compared to controls, correlating with the number of switched memory B cells and with several markers of disease severity (89, 91, 95, 100–104). Moreover, Tfh cells have been also investigated at tissue level in SLE: Tfh-like cells are present in inflamed kidney of patients with nephritis (105, 106), where they cluster with B cells building structures similar to ectopic GCs (106).

The role of Tfr in SLE remains unclear and literature data are inconsistent. Some authors observed a reduction in the frequency of circulating Tfr cells in SLE patients (107, 108) with an increase in the ratio Tfh/Tfr, associated with a negative correlation with disease activity and titers of anti-dsDNA antibodies (107). On the contrary, another study revealed a significant increase of Tfr cells in peripheral blood with an increase in the Tfr/Tfh ratio (109). Discrepancies could be related to the strategy used to define Tfr cells by flow cytometry (CD4⁺CD25⁺CD127^{low-intermediate}CXCR5⁺

(107) or CD4⁺CXCR5⁺FoxP3⁺ (109)), as well as the different recruitment of subjects within each study.

T follicular helper cell expansion and aberrant GC responses play clearly a crucial role in the pathogenesis of lupus in mice and data obtained so far in human SLE seem to go in the same direction. Tfh cells could, therefore, represent an interesting therapeutic target. Conventional treatments used in SLE (steroids, immunosuppressive drugs) result in a decrease in the number of activated cTfh cells (90, 91), associated with a clinical improvement (90). A treatment with low-dose recombinant human IL-2 in SLE patients leads also to a decrease in the relative number of cTfh cells (110). However, the specific targeting of these cells requires a better understanding of the mechanisms involved in their aberrant activation during SLE. In this way, OX40 ligand (OX40-L)–OX40 axis has been identified as a key player in the increased Tfh responses (111): OX40-L, highly expressed by myeloid APCs in active SLE patients in response to TLR7 activation by RNP-anti-RNP immune complexes, promotes the differentiation of CD4⁺ T cells in functional Tfh cells (111). In this way, in human SLE, the OX40-L–OX40 axis provides an amplification loop in the generation of autoantibodies (112) and could represent an attractive pathway to target. However, although anti-OX40 and anti-OX40L antibodies exist, there is for instance no ongoing trial of these molecules in human SLE.

RHEUMATOID ARTHRITIS (RA)

Rheumatoid arthritis is a systemic auto-immune disease, associated with acute and chronic synovial inflammation, cartilage lesions, and bone erosion (113). Various autoantibodies associated with RA and which play important roles in the pathological progression of the disease have been identified, including rheumatoid factor and antibodies against cyclic citrullinated peptides (anti-CCP). Exact pathogenic processes of RA are not totally understood and involve many types of immunocompetent cells. Nevertheless, interactions between T and B cells are required for antibody production and, similarly to what are observed during SLE, many of the RA-related autoantibodies are high-affinity IgG antibodies, indicative of the involvement of GC reactions and, therefore, suggesting a critical role of Tfh cells in the disease pathogenesis.

Data from murine models of arthritis seem to indicate that some essential Tfh cells associated molecules are required for the disease development. For example, in the model of collagen-induced arthritis (CIA), CXCR5 was identified as an essential factor for the induction of the inflammatory autoimmune arthritis, as CXCR5-deficient mice and mice selectively lacking CXCR5 on T cells are resistant to CIA (114). In another model (transfer of self-reactive CD4⁺ cells from KRN-TCR transgenic mice into recipient animals), Chevalier et al. observed that deficiency in CD4⁺ cells of signaling of lymphocytic activation molecule-associated protein (SAP) (a protein known to promote Tfh cell differentiation during GC formation) protects mice from the induction of arthritis, indicating that long-lasting interactions between T and B cells and GC formation are required for the development of the disease (115).

In human RA, it is worth noting that literature data are conflicting regarding Tfh cells frequencies (Table 2). In five studies, it was observed increased frequencies of cTfh cells in patients as compared to controls (116–120), especially in those with new-onset disease. For some authors, this increase is constitutive and is not associated with the activity of arthritis, as both patients with active or inactive disease have higher frequencies of activated cTfh cells and of subsets with B cell helper phenotype (Tfh2 and Tfh17) (116). Still these findings contrast with other reports where percentages of cTfh cells have been associated with the disease activity [mostly assessed by 28-joint count disease activity score (DAS28)] (117–119, 121) and with the levels of autoantibodies (119–121). An enrichment of Tfh cells was observed in synovium tissues of patients with RA whereas they were absent in both osteoarthritis and normal synovium tissues (122, 123). By contrast of the five studies mentioned above, three other ones dismiss the presence of increased frequencies of Tfh cells during

RA. No differences were found in the percentages of cTfh cells or within Tfh1, Tfh2, and Tfh17 subsets in RA patients, as compared to healthy controls or patients with undifferentiated arthritis, without correlation with DAS28 (124, 125) and Penatti et al. even observed lower frequencies of ICOS⁺ cTfh cells in patients with RA (122).

Investigations on the implication of Tfh cells in the pathogenesis of the disease have also led to the evaluation of the impact of their signature cytokine, IL-21. RA patients have higher serum levels of IL-21 as compared to controls (117, 120, 121), correlating with DAS28 (117, 121), serum anti-CCP antibodies, and frequencies of cTfh cells (121). IL-21R is highly expressed in inflamed synovial tissues of RA patients (126, 127), by macrophages but also by fibroblasts with activated phenotype (126). IL-21 has an impact on local T-cell activation and proliferation, but also promotes the aggressive migration, invasion, and metalloproteinase secretion by fibroblast-like synoviocytes (128). In RA synovial cell cultures, neutralization of IL-21 and IL-15 inhibits their pro-inflammatory cytokine production (129). Finally, in the K/BxN mouse model of RA, IL-21R deficiency is sufficient to protect from arthritis (130).

Potential use of Tfh cells as biomarkers of RA has conducted to the evaluation of the impact of treatments on these populations. In 2013, Wang et al. observed a significant reduction in the percentages of ICOS⁺ Tfh cells following 1 month of disease-modifying antirheumatic drugs (DMARDs) and *T. wilfordii* (Chinese herb) in the drug-responding patients (119). On the contrary, no decrease in the frequency of Tfh cells in response to 24 weeks of DMARD therapy (mostly methotrexate monotherapy) was observed, whereas IL-21 concentrations were remarkably reduced (117). In patients with RA treated by abatacept, which modulates CD28-mediated T cells co-stimulation, the treatment is associated with a marked decrease in the proportion of activated cTfh (118). Data from murine model of RA associated with a breach of self-tolerance brought some mechanistic explanations on this observation made in RA patients: abatacept is associated with a failure of T cells to acquire a follicular helper cell phenotype (CXCR5⁺ICOS⁺), to proliferate, to enter B cell follicles, leading to a reduction in antibodies responses (131).

It is known that environmental factors are implicated in the development of arthritis and two recent studies suggest that generation of RA-related Tfh cells possibly relies on gut microbiota. Using the K/BxN autoimmune arthritis model, Teng et al. evaluated the role of a specific type of commensal gut bacteria, named segmented filamentous bacteria (SFB), in the regulation of Tfh cell responses (132). SFB are known to drive autoimmune arthritis in this mouse model (133) and it was shown that SFB induce an increase in the systemic Tfh cell populations by driving differentiation and egress of Tfh cells from intestinal Peyer's patches into systemic lymphoid sites, leading to an increase in auto-antibody responses and to the development of arthritis (132). Using the same murine models, Block et al. confirmed the requirement of gut microbiota in the differentiation of Tfh cells, the formation of GCs, the auto-antibody production, and the development of the experimental arthritis. Depletion of gut microbiota in the animals, by the use

TABLE 2 | T follicular helper (Tfh) cells in human RA.

Study	Patients (n)	
Circulating Tfh		
Arroyo-Villa et al. (116)	34	Expansion of cTfh cells
Zhang et al. (117)	54	Expansion of cTfh cells Correlation with disease activity (values of DAS 28)
Nakayamada et al. (118)	108	Expansion of cTfh cells in patients with active RA Correlation with autoantibody titers Abatacept treatment resulted in decrease in the proportion of activated cTfh cells
Wang et al. (119)	25	Expansion of cTfh cells Correlation with disease activity (values of DAS 28)
Ma et al. (120)	53	Expansion of cTfh cells Correlation with autoantibody titers (level of anti-CCP Ab)
Liu et al. (121)	120	Expansion of cTfh cells Correlation with disease activity (values of DAS 28) and with the titers of autoantibodies (anti-CCP Ab levels)
Penatti et al. (122)	25	Lower frequencies of cTfh cells ICOS+ in patients with RA
Costantino et al. (124)	24	No differences in cTfh cells between patients with RA and healthy controls or patients with undifferentiated arthritis
Chakera et al. (125)	35	No differences in cTfh cells between patients with RA and healthy controls
Synovium tissues		
Penatti et al. (122)	25	In the synovial compartment of RA patients, enrichment in Tfh cells as compared to patients with osteoarthritis
Chu et al. (123)	2	Localization of ICOS+ Tfh cells in the RA synovium tissues, as compared to osteoarthritis synovium or normal synovium tissues where they are absent (triple-fluorescence immunostaining and confocal laser scanning)

Ab, antibody; Anti-CCP, antibodies against cyclic citrullinated peptides; cTfh cells, circulating Tfh cells; CXCR5, chemokine (C-X-C motif) receptor 5; DAS, disease activity score; ICOS, inducible co-stimulator; RA, rheumatoid arthritis.

of antibiotics, reduced the number of Tfh cells and the levels of antibodies (134).

Finally, we would like to highlight the results published recently by Rao et al. in *Nature*, who identified a novel subset of T cells in the synovium of patients with RA (135). Using multidimensional cytometry, transcriptomics, and functional assays to analyze activated T cells in joint tissues, authors observed, in seropositive RA patients, abundant populations of a specific subset of T cells, defined as PD-1^{hi}CXCR5⁺CD4⁺ and which they named “peripheral helper” cell population. This population, not expanded in patients with seronegative RA and of whom frequencies are highly correlated with disease activity in response to therapy, shares B-cell-helper functions with Tfh cells but seems to differ from them by a specific migratory program targeting inflamed tissues rather than lymphoid tissues (135). These results need to be confirmed and some questions need to be addressed, but this study raises the possibility of the existence in RA of a subset with high pathologic abilities that could be of great interest in a therapeutic perspective.

T follicular helper cells have also been investigated in patients with ankylosing spondylitis (AS), another rheumatologic disease, with contradictory results. Higher frequencies of cTfh cells have been observed in AS patients as compared to controls, associated with elevated concentrations of serum IL-21 (136, 137), whereas Bautista-Caro et al. reported decreased frequencies of cTfh cells, plasmablasts and underrepresentation of cTfh subsets with a B helper phenotype in patients with AS naïve from TNF blockers (138). Frequencies of Tfr cells were also reported to be significantly higher in AS patients than in healthy controls, with higher ratio of Tfr/Tfh cells (137).

SJÖGREN SYNDROME

Primary Sjögren's syndrome (pSS) is a systemic autoimmune disease, presented as a disabling sicca syndrome associated with asthenia and pain (139). It is characterized by lymphocytic infiltration and destruction of exocrine glands, primarily the lacrimal and salivary ones. Crucial role of B cells in pSS pathogenesis is illustrated by the emergence of circulating immune complexes, autoantibodies, ectopic GCs in the affected tissues, and enhanced risk of developing B cell lymphoma (140) underlying a possible important role played by Tfh cells in the pathogenesis of this disease. Increased percentages of cTfh cells (especially cTfh17 subset) have been observed in peripheral blood of patients with pSS (141, 142). This increase seems to be limited to pSS patients with severe manifestations of the disease. Indeed, in patients with pSS who present extraglandular manifestations (EGMs), Szabo et al. observed an increase in activated cTfh cells (ICOS⁺ or PD1⁺ cTfh) as compared to pSS patients without EGMs and to healthy controls, whereas there was no difference in cTfh percentages between patients without EGMs and controls (143). Elevated Tfh percentages were also observed in the anti-SSA/SSB positive patients (143). Similarly, a significant elevation in the proportion of IL-21-producing PD1⁺ cTfh cells was described in pSS patients with EGMs and in patients with autoantibodies against anti-SSA/Ro (95). More interestingly, correlation between cTfh cells percentages and importance of glandular

involvement assessed by the focus scores of labial salivary gland biopsies has been estimated with a coefficient R equal to 0.6984 (143). Otherwise, increased serum levels of IL-21 are observed in pSS patients with a significant association with the systemic DAS28 (ESSDAI) (144, 145). Regarding salivary glands, Jin et al. reported increased numbers of Tfh cells in pSS patients as compared to controls (141). When analyzing the expression of cytokines and transcription factors associated with the different Th subsets (Tfh, but also Th1, Th2, and Th17) in labial salivary glands, Maehara et al. observed an increase in the expression of all Th subset-related molecules in pSS patients as compared to controls. However, expression of Tfh-related molecules was associated with a strong lymphocytic infiltration and especially in the presence of GCs (146). Differentiation of naïve CD4⁺ T cells seems to be particular during pSS and implies the interactions with salivary gland epithelial cells such as IL-6 and ICOS-L expressed by these cells contribute to the direct induction of Tfh cells differentiation (145).

Frequency of cTfh cells in pSS patients is almost reduced by 50% after B cell depletion therapy and reach levels comparable to controls, in association with a significant lowering of serum levels of IL-21, with a decrease in anti-SSA/Ro and antiSSB/La antibodies and with the improvement of the disease activity measured by ESSDAI (147). During B cell repopulation, frequencies of cTfh cells return to baseline levels. Like described in RA, specific effects of abatacept on Tfh cells are observed during pSS treatment (148). Abatacept results in the predominant reduction of percentages and numbers of cTfh cells (other effector subsets are not affected, except Treg cells), of serum levels of IL-21 and of autoantibodies. A decrease in ICOS expression on CD4⁺ T cells is observed in both peripheral blood and parotid gland tissue and this phenomenon correlates significantly with a lowering in the DAS28 (148).

NEUROLOGICAL DISEASES

Multiple Sclerosis (MS)

Multiple sclerosis is a chronic, inflammatory, and autoimmune disease affecting the central nervous system (CNS), leading to the destruction of myelin and axons (149). In pathological studies, important meningeal inflammation with ectopic lymphoid follicles, B and plasma cells is observed (150, 151). This suggests a possible implication of T helper cells and especially Tfh cells in the pathogenesis of the disease. In peripheral blood from patients with relapsing–remitting or secondary progressive forms of the disease, an increase in the frequencies of ICOS⁺ cTfh cells is observed, associated with an increased expression of Tfh and plasmablast markers by cerebrospinal fluid (CSF) cells (152). Activated memory cTfh cells (CCR7⁺ICOS⁺) were also found increased in another study in patients with relapsing MS. Moreover, they observed increased levels of plasma and CSF IL-21 correlating positively with the score of severity of the disease (EDSS score) and with levels of auto-antibodies directed against myelin basic protein or myelin oligodendrocyte glycoprotein (153). Treatment with systemic steroids (methylprednisolone) leads to a decrease in the numbers of activated cTfh cells and in the plasmatic levels of IL-21 (153).

Neuromyelitis Optica Spectrum Disorders (NMOSDs)

Neuromyelitis optica spectrum disorders are also inflammatory demyelinating diseases affecting CNS, characterized clinically by attacks of myelitis and optic neuritis and biologically by the presence of highly specific and pathogenic autoantibodies directed against the extracellular domain of the water channel protein aquaporin-4 expressed in astrocytes (NMO-IgG) (154). Activated Tfh cell frequencies are higher in NMOSD subjects, as compared to healthy controls and even as compared to MS patients and are associated with disease activity and with increased levels of plasma and CSF IL-21 (155, 156). Treatment with methylprednisolone is associated with a significant decrease in the proportion of cTfh cells in NMOSD patients (155).

Myasthenia Gravis (MG)

Myasthenia gravis is characterized by the production of anti-acetylcholine receptor (AChR) antibodies, leading to a dysfunction of the neuromuscular junction and *in fine* to muscle weakness (157). Ocular and generalized MG are the two major clinical forms of the disease. Expansion of cTfh cells, concomitant with expansion of circulating plasmablasts, is observed in patients with MG as compared to healthy control subjects, and is associated with the clinical severity and the form of the disease (158–161). Strong correlations between cTfh cells and titers of anti-AChR autoantibodies are frequently described (159–161). On the contrary, proportions of Tfr cells are decreased in MG patients as compared to controls, leading to an imbalance in the Tfr/Tfh ratio (159, 162). One important specificity of MG is the frequent association with thymoma and the use of thymectomy as a treatment, leading to the possible pathological analysis of thymus obtained from MG patients. Clinical severity of the disease (subjects with generalized MG versus subjects with ocular MG and controls) is correlated with higher mRNA expression of four markers by thymoma cells (CXCR5, Bcl-6, ICOS, and IL-21) (163). Higher percentages of thymic Tfh cells are present in MG patients, as compared to patients with thymoma without MG or patients with healthy thymus (control thymic biopsies from cardiac surgery cases) (164).

IgG4-RELATED DISEASE (IgG4-RD)

IgG4-related disease consists in lymphoplasmacytic infiltrates of CD4⁺ T cells and IgG4⁺ plasma cells associated with fibrosis. It affects mainly males over 50 years and may involve all tissues (pancreatitis, retroperitoneal fibrosis...) (165). IgG4-RD patients are characterized by higher proportions of cTfh cells, as well as increased proportions of plasmablasts as compared to controls (166). The two populations correlate positively within each other and cTfh cell rates are positively correlated with EGMs of IgG4-RD (166). Proportions of cTfh cells that express a high level of PD-1 positively correlate with serum levels of IgG4, IgG4/IgG ratio, and number of involved organs (167). When cTfh subsets are analyzed, a significant specific expansion of Tfh2 cells is observed, associated with serum levels of IgG4, IgG4/IgG ratio, number of plasmablasts, disease activity, and number of affected organs

(168–170). Tfh2 cells appear to be responsible, in the pathogenesis of the disease, for the induction of the differentiation of naïve B cells into plasmablasts and of the increase in the production of IgG4 in patients with active disease (169). Contradictory results have been published regarding the population of Tfh1 cells: a Japanese team observed an increase in the number of activated Tfh1 cells in IgG4-RD, correlating with disease activity but not with serum IgG4 levels, whereas another team reported a decrease in the proportion of cTfh1 cells in patients as compared with controls (170).

One particularity of IgG4-RD is the existence of numerous data with histopathological examination. In fact, in IgG4-RD, it is necessary to get a biopsy-proven diagnosis, allowing the analysis of Tfh cells directly in the involved tissues. Histological data reveal marked Tfh cell infiltration or overexpression of Tfh-related molecules in the different affected organs studied (166, 171–173), colocalizing with B cells and plasma cells (171). Tfh cells infiltrating the submandibular glands (SMGs) of patients suffering from IgG4 dacryoadenitis and sialadenitis are characterized by high expression levels of Bcl6, PD-1, and ICOS, as compared with SMGs from control patients with head and neck cancers, and functional analysis reveal that they have a higher capacity than tonsillar Tfh cells to help B cells to produce IgG4 (167). Expression of IL-21 in labial salivary glands from patients with IgG4-RD correlates with the number of GCs and with the IgG4/IgG ratio (172). All teams working on Tfh cells and IgG4-RD showed a decrease in Tfh percentages in patients treated by steroids or rituximab, correlating to the improvement of symptoms (166, 167, 169, 170).

VASCULITIS

Large Vessels Vasculitis

To our knowledge, role of Tfh cells in the physiopathology of large vessels vasculitis has been rarely investigated. One team studied Tfh cells in granulomatosis with polyangiitis (GPA) (174), disease characterized, in the majority of patients, by the development of ANCA autoantibodies with specificity for PR3 (ANCA/anti-PR3). Because GPA has benefited recently from B-cell depletion therapy, it raises the question of the underlying mechanism of its effectiveness. Authors found, in GPA patients on conventional therapies, an increased frequency of cTfh cells. This increase is not observed in GPA patients treated with rituximab who are clinically improved and in whom cTfh cells frequencies are statistically not different from those seen in healthy controls (174).

Small Vessels Vasculitis

Ig A vasculitis, also called Henoch Schönlein Purpura (HSP), is the most common small vessel vasculitis which affects particularly children. Vascular deposits of IgA-related immune complexes characterize this systemic inflammatory disease. Circulating Tfh cells are increased in children with acute HSP as compared to healthy controls and are associated with higher levels of IL-21 (175, 176). Levels of cTfh and IL-21 lowered significantly following disease remission (175). Adult patients with HSP nephritis (HSPN) display same features, with increased

levels of cTfh cells and IL-21 as compared to healthy controls and a significant reduction after treatment (177). When further investigating Tfh subpopulations, an increase in cTfh2 and cTfh17 is present in HSP patients compared to healthy controls and their levels positively correlated with serum IgA ones (178). The increases in cTfh2 and cTfh17 cells counts are abrogated by treatment.

IDIOPATHIC THROMBOCYTOPENIC PURPURA (ITP)

Idiopathic thrombocytopenic purpura is characterized by a low platelet count, which is the result of both insufficient platelet production and increased platelet destruction by auto-antibodies directed against platelet glycoproteins. Main antibody isotype in ITP is represented by IgG, which underlies a class-switch recombination, a mechanism supported by Tfh cell help to B cells. In adult ITP patients, there is an increase in the proportion of cTfh cells with high expression of ICOS or PD-1 (179). This is particularly marked in the sub-group of patients with positivity of anti-platelet antibodies as compared to platelet-antibody-negative patients. Plasma levels of IL-21 are also significantly increased in patients with active ITP as compared to controls (179, 180). In the pediatric population, which is frequently impacted by ITP, similar results are observed: frequencies of cTfh cells are markedly increased during ITP, with a strong negative correlation between the proportion of cTfh cells and platelet count, as well as with increased serum levels of IL-21 (181). It should be highlighted that the frequencies of cTfh cells return to normal levels after therapy (intravenous Ig, corticosteroids, or both) in patients with newly diagnosed ITP, whereas children who fall in chronic ITP (cITP) have a persistent increase in both cTfh cell frequencies and IL-21 levels. Moreover, in cITP subjects, overexpression of ICOS-L by CD19⁺ B cells remains after treatment, while it markedly decreases in the other group (181).

Spleen is the site of platelet destruction by splenic macrophages phagocytosis and as it is the primary site for the activation of B cells and for the autoimmune response and, therefore, an organ of interest in ITP. Splenectomy is one possible therapeutic option for resistant ITP and pathological analysis of the resected organs gives precious information. Audia et al. characterized Tfh cells in the spleen of 13 ITP patients compared to 8 controls (splenectomy because of spleen traumatism) (182). Splenic Tfh cells frequencies (CXCR5⁺ICOS⁺PD-1^{hi}) are increased during ITP, concomitant with an expansion of GCs and with an increase in splenic CD38⁺ B cell subsets (pre-GC B cells, GC B cells, and plasma cells). Furthermore, IL-21 expression in splenic CD4⁺ T cells correlates with Tfh cell abundance. Finally, *in vitro* stimulation of B cells with IL-21, in the presence of CD40 engagement, induces the differentiation of B cells in antiplatelet antibodies secreting plasmablasts in ITP patients (182). The expansion of splenic Tfh cells is dramatically decreased after B cell depletion induced by rituximab, associated with a decrease in the absolute count of cTfh cells, even if the therapy is not clinically effective (183).

CHRONIC INFLAMMATORY SKIN DISEASES

Psoriasis

Skin lesions in psoriasis are thought to be due to a deregulated interplay between immune cells and keratinocytes, leading to proliferation of keratinocytes in the interfollicular epidermis, inflammation of the stratum corneum, dermal angiogenesis, and infiltration with mononuclear cells. Circulating Tfh cells are increased in blood from patients with psoriasis vulgaris as compared to controls, both from a numerical but also from an activation point of view, and frequencies of some activated counterparts of cTfh cells are correlated with disease severity [Psoriasis Area and Severity Index (PASI) score] and with the accumulation of activated B cells (184, 185). Regarding cTfh cells subpopulations, psoriatic patients are characterized by a significant increase in cTfh17 rates, with a trend of increasing frequency of cTfh2 cells and decreasing frequency of cTfh1 cells, leading to an increase in (Tfh17⁺Tfh2⁺)/Tfh1 ratio (186). Frequency of cTfh17 correlates with the PASI score and decreases with the improvement of the skin disorder (186). Frequencies of cTfh cells are reduced after 1 month of acitretin treatment (184). In psoriatic skin lesions, Tfh are found in great amount contrary to healthy skin tissues or non-lesional skin tissues of psoriasis (184).

Serum levels of IL-21 are higher in patients with psoriasis than in controls and positively correlate with PASI score (185). Moreover, high levels of IL-21 protein and IL-21 mRNA are observed in lesional psoriasis skin compared to samples taken from non-lesional skin of the same patients and from healthy controls (187).

Atopic Dermatitis (AD)

Because of an increased level of serum IgE in AD, some authors have focused on Tfh cells in this disease. There is an increased level of ICOS⁺Tfh and ICOS⁺PD1⁺ Tfh cells in children with AD when compared to adults with AD and to controls. Despite no difference in serum levels of IL-21, absolute numbers of IL-21-producing Tfh cells are significantly expanded in children with AD, and correlate to disease activity (measured by the SCORAD index) (188).

Pemphigus and Bullous Pemphigoid

Pemphigus is an autoimmune disease with antibodies directed against the desmosomal cadherin Desmoglein (Dsg) 3 and Dsg1 that cause loss of keratinocyte adhesion in the human skin. There is an increase in percentage of total cTfh in peripheral blood of patients with pemphigus, along with elevated levels of serum IL-21, despite no difference in ICOS⁺ or PD1⁺ Tfh cells between patients and healthy controls (189). By stimulating PBMC from pemphigus patients and controls with Dsg3 protein *ex vivo*, authors identified autoreactive IL-21-secreting cells in 50% of the pemphigus patients.

Bullous pemphigoid is another autoimmune skin disease, with antibodies directed against hemidesmosomal proteins within the dermal–epidermal junction [non-collagenous 16A domain (NC16A) and transmembrane domain of the hemidesmosomal

protein (BP180)]. Increased level of IL-21 and increased frequency of ICOS⁺ and PD1⁺ cTfh cells are positively correlated with high levels of serum anti-BP180-NC16A antibodies, which have been recognized as a serum marker of disease severity. Levels of serum IL-21 and Tfh rates are significantly reduced after efficient therapy with methylprednisolone (190).

ENDOCRINOPATHIES

Type 1 Diabetes (T1D)

Type 1 diabetes is due to the destruction of insulin-producing- β -cells in the pancreas. Despite being considered as a T-cell-driven disease, a pathogenic role of B cells producing autoantibodies against β -islets has been proven. Using a transgenic mouse model of diabetes, Kenefeck et al. identified a Tfh signature (that is a strong up-regulation of Tfh cell genes) in the islet-specific T cells responding to pancreatic antigen, as compared to T cells sorted from inguinal lymph nodes (191). This was confirmed at the protein level by flow cytometry. Interestingly, transfer of T cells with a Tfh cell phenotype (obtained from pooled pancreatic lymph nodes from diabetic mice and enriched according to CXCR5 expression) transferred diabetes to recipient animals more efficiently than CXCR5-depleted T cells (191).

At the transcriptomic level, memory CD4⁺ T-cells from T1D patients (with a mean duration of T1D of 19 years) overexpress Tfh cell genes (*CXCR5*, *ICOS*, *PD-1*, *Bcl6*, and *IL-21*) (191). Rates of cTfh cells are increased in patients with T1D, as compared to controls (192–194). This increase is associated with an enhanced IL-21 production by memory CD4⁺ cells in patients (192, 193). Interestingly, authors have shown an increase in activated cTfh (CXCR5⁺PD1⁺ICOS⁺) cells in newly diagnosed T1D children and in at-risk children with impaired glucose tolerance compared to aged-matched healthy children (194). A longitudinal analysis of at-risk children showed an increased rate of Tfh just before progression to clinical T1D, whereas children who did not progress had a stable Tfh frequency. Comparison between diabetic patients with ≤ 1 autoantibody and diabetic patients with ≥ 2 antibodies showed a strongly increased frequency of activated cTfh cells in the second group. It suggests that multiple autoantibody-positivity identifies a subgroup of patients with increased Tfh activation at disease presentation (194).

PD1⁺ Tfh cells positively correlate with blood glucose levels and negatively to the Glomerular Filtration Rate (eGFR) in patients with diabetic nephropathy (195). Besides, frequency of ICOS⁺ Tfh cells seems to inversely correlate with the concentrations of fasting serum C-peptide, while after 4 months of rituximab therapy, 50% of patients increase their levels of this marker, parallel to the decrease in Tfh percentages (192).

Autoimmune Thyroid Disease

Elevated percentages of ICOS⁺ and PD1⁺ Tfh cells are present in patients with autoimmune thyroid diseases [Grave disease (GD) or Hashimoto's thyroiditis (HT)]. ICOS⁺ cTfh cells correlate positively with the serum concentrations of autoantibodies directed against anti-TSH receptor, thyroperoxidase, or thyroglobulin (196).

Analysis of thyroid tissues is sometimes possible in these diseases: both GD and HT are characterized by an increased lymphocytic infiltration (196–198), with frequent GCs formation. Tfh cells are detected *in situ*, in HT (196) and GD (197). Expression of Tfh-related molecules (IL-21/IL-21R, CXCR5/CXCL13) is detected in GD thyroid tissues (197), with a positive correlation between the expression of IL-21 mRNA in GD thyroid tissues and the serum levels of autoantibodies. mRNA expression of CXCR5 and CXCL13 is correlated with the number of lymphocytic infiltrates and ectopic GCs in thyroid tissue from patients affected by HT or GD (198).

HEPATOLOGICAL AND GASTROENTEROLOGICAL DISEASES

Autoimmune Hepatitis (AIH)

Autoimmune hepatitis displays varied manifestations from asymptomatic, mild chronic hepatitis to acute-onset fulminant liver failure. Studies with murine models of AIH have raised a possible crucial role of Tfh in the disease (199, 200), by showing that dysregulated Tfh cells in the spleen are responsible for the induction of fatal AIH. In humans, percentages of activated cTfh cells (PD-1⁺ or ICOS⁺ Tfh cells) are increased in patients with AIH compared to controls (201, 202). Frequency of activated cTfh cells is positively correlated with serum IgG levels (201, 202) and negatively with serum albumin and serum prothrombin time (201). This population decreases significantly consecutively to prednisolone treatment, with the decrease of serum alanine transferase (201). Serum levels of IL-21 are higher in patients with AIH than in patients with other liver diseases or healthy volunteers (202, 203), correlating with the numbers of cTfh cells. They are associated with the severity of the disease. Finally, they correlate positively with total serum bilirubin levels and negatively with serum albumin (203). Data obtained from immunohistochemistry studies and from flow cytometry on extracted cells from liver biopsies of patients with AIH have been also published: frequencies of activated Tfh cells are significantly increased in the liver from patients and positively correlate with their circulating counterparts in blood of patients (201). A positive correlation of serum IL-21 levels and the grading of necro-inflammatory activity is also described (203).

Primary Biliary Cholangitis (PBC)

Circulating Tfh (201), ICOS⁺ cTfh (204) and PD1⁺ cTfh cells (205, 206) are increased in patients with PBC as compared to controls, in association with a decrease in Tfr cells and in consequence in the Tfr/Tfh ratio (206). Levels of cTfh cells are even higher than in AIH patients (204) and functional capacities of cTfh cells from PBC patients are greater than those from controls (204). cTfh frequencies correlate with disease severity (204). They are higher in patients with cirrhosis (non-decompensated or decompensated) than in the non-cirrhotic group, and higher in the group of patients with anti-mitochondrial antibodies than in the group without (205). In patients responders to the classic treatment of PBC (ursodeoxycholic acid), there is a decrease in cTfh rates and a trend to a lower production of IL-21 by Tfh cells (204). As in AIH,

histological analysis of liver samples reveals an accumulation of PD1⁺Bcl6⁺ Tfh cells around the damaged interlobular bile ducts in PBC with chronic non-suppurative destructive cholangitis; these infiltrating Tfh cells organize follicle-like structures and collocate with B cells around the bile ducts (204). Similar increases of Tfh cells are also present in spleen samples of patients with PBC, as compared to controls (traumatic splenectomy) (204). The aberrant Tfh cell activation in PBC patients can possibly rely on abnormal response toward bacterial antigens stimulation (207).

Inflammatory Bowel Diseases (IBD)

Circulating Tfh cells have also been studied in patients with IBD [Crohn's disease (CD) or ulcerative colitis (UC)], showing an increase in their proportions in IBD patients as compared to healthy controls (208, 209), with a significant specific increase in Tfh1 and Tfh17 subsets (209) and a reduction in the frequency of cTfh cells (208). Levels of cTfh cells are associated with symptoms of severity of IBD: they are significantly elevated in the penetrating form of CD, as compared to the inflammatory or stricturing ones (209). In UC, the values of Mayo clinic score (measuring disease severity) and of C-reactive protein positively correlate with cTfh cells and serum IL-21 levels (208). Because Tfh cells may affect the progression of various cancers and because CD patients are at risk of colorectal cancer, the question has been raised to compare cTfh levels in CD patients with or without neoplasia. Patients who developed a colorectal cancer, compared to those who did not, have a significant increase of cTfh cells (209). In colonic tissue sections of patients with UC, proportion of Tfh cells among CD4⁺ T cells is increase (210).

Specific contribution of IL-21 to the pathogenesis of IBD has been studied. It has been observed enhanced serum levels of this cytokine in patients (208), an increase in its expression in UC colonic tissues (210) and interesting findings indicate an important role of CD4⁺ T intestinal lamina propria lymphocytes in the production of IL-21, especially after their activation by IL-12 (211).

COMMON VARIABLE IMMUNE DEFICIENCIES (CVID)

Common variable immune deficiencies is the most symptomatic primary immunodeficiency and manifests by recurrent respiratory and gastrointestinal tract infections. This disease is heterogeneous and diagnosis criteria, as defined by European society for immunodeficiencies, include onset after 2 years old, deficit in serum Ig (multiple classes) not explained by other known causes and impaired vaccination responses (212). Impaired B cell differentiation is a hallmark of the disease and, despite normal levels of total B cells in most cases, patients harbor lower levels or absence of smB cells (213). Based on these alterations, the European multicenter trials proposed a classification called EUROClass which hinged on levels of circulating B cells, of smB cells and on the presence of an expansion of transitional B cells or of CD21^{low} cells (214). In more than half of the patients, CVID causes inflammatory disorders, such as lymphoproliferation, granulomatous disease, malignancy, and autoimmunity on

the top of the immunodeficiency leading to infection susceptibility (215). These complications cause increased morbidity and mortality. More than 25% of CVID patients have autoimmune complications (215). ITP and autoimmune hemolytic anemia are the most frequent autoimmune disorders but numerous others, such as vitiligo, pernicious anemia, SLE, RA, antiphospholipid syndrome, anti-IgA Ab disease, juvenile idiopathic arthritis, Sjogren's syndrome, psoriasis, thyroiditis, uveitis, and vasculitis can also be found in CVID patients (215). These observations highlight a paradigm in CVID pathogenesis: despite a defect in B cell differentiation and in serum Ig, patients develop autoantibodies and harbor autoimmune complications. Mechanisms responsible for this paradigm may highlight failures in specific checkpoints for autoreactive B cells and are yet to be clearly identified. Interestingly, Patuzzo et al. (216) have shown an increase in the CD21^{low} population in CVID patients with ITP. CD21^{low} cells may develop under chronic inflammatory conditions from memory B cells and are present in high levels in autoimmune patients including ones affected by ITP, pSS, and SLE. Therefore, one can hypothesize a role of these CD21^{low} smB cells in the development of autoimmune complications observed CVID patients. Nevertheless, further experiments are needed to explore this possibility.

CD4⁺ T cells play a central role in B cell differentiation into memory and Ig-producing cells. It is, therefore, not surprising to observe abnormalities of the T cell compartment in CVID patients. Patients have usually lower levels of CD4⁺ T cells and normal number of CD8⁺ T cells. Genetic defects in the T cell compartment may be detected. One example is the discovery of ICOS mutations in some patients. ICOS has a key role in GC reactions and in smB cell generation and as mentioned earlier, most CVID patients have defects in this B cell population. Grimbacher et al. have identified homologous deletion of *ICOS* genes in CVID patients causing a failure in ICOS expression on T cells (217, 218). These patients have impaired GC formation and defects in class-switching leading to hypogammaglobulinemia and, therefore, defective T cell helping in late B cell differentiation. Combining clinical features of the nine ICOS-deficient patients allow manifestation of the full range of non-infectious related complications. Nevertheless, four of these patients do not have autoimmune diseases. Interestingly, these patients also present lower levels of circulating CXCR5⁺ Tfh cells despite normal numbers of CD4⁺ T cells (15). Among the CD4⁺ T cells, Tfh cells play a critical role in B cell differentiation especially for smB cell differentiation. It seems critical to assess the potential role of these cells in CVID pathogenesis. Our group (submitted manuscript) and others observed an increase of circulating Tfh cells in CVID patients (219–221). Interestingly, patients with non-infectious complications or classified as smB based on the EUROClass harbor increased cTfh (220) which is even more pronounced in the smB- CD21^{low} subgroup (221). Tiller et al. demonstrated in 2007 (222) that the smB cell population (IgG⁺) contains some autoreactive B cells in normal adults. It is then possible that smB cells in CVID patients, despite their low levels, contribute to autoimmunity. Consequently, Tfh cells could be a part of the immune responses leading to autoimmune manifestations observed in CVID patients considering their central role in

smB cell differentiation. Moreover, as mentioned earlier, CD21^{low} memory B cells are increased in several autoimmune contexts (216). Still, when we compared patients with autoimmune complications with patients harboring other type of comorbidities, we could not detect any significant differences in levels of Tfh or Tfh subtypes (submitted manuscript). It will be then necessary to perform further experiments to assess the functions of Tfh cells in CVID patients and to evaluate their impact on autoreactive Ab secretion.

Regarding Tfh cell subpopulations, we (submitted manuscript) and others (220, 221) highlight specific increases of the cTfh1 in non-infectious only CVID patient blood. By contrast, cTfh17 cells were decreased. Moreover, Tfh cells from CVID patients expressed elevated levels of IFN γ (220, 221) and sera from patients contained higher concentrations of IFN γ compared to healthy donors (221). Finally, we and Unger et al. showed respectively higher levels of CXCR3 and T-bet, markers of Th1-oriented cells, in SLO from CVID patients. Altogether, these data demonstrated an imbalance in Tfh subpopulations in CVID patients harboring non-infectious complications in favor to cTfh1. These data suggest an IFN γ -enriched environment during B cell differentiation/maturation in CVID patients. Cols et al. have associated elevated IFN γ levels with manifestations of complications in CVID patients and expansion of type 3 innate-lymphoid cells (ILCs) (223). Friedmann et al. (224) confirmed an imbalance in circulating ILC2/ILC3 cells in CVID patients but argued that given the relative abundance of Th1CD4⁺ T cells ILC would not be the main source of IFN γ in CVID. Unger et al. showed that exogenous addition of IFN γ in B/T cocultures reduces IgG and IgA production (221). Still, the impact of IFN γ on CD21^{low} cell generation and/or on autoreactive B cell activation was not directly addressed in CVID and, therefore, is yet to be determined. In conclusion, evidences from the literature strongly suggest a role for Tfh in pathogenesis of the more severe forms of CVID, but not limited to the autoimmune disorders observed in some of these patients. Experiments are still needed to determine Tfh implication in CVID and the possible role of IFN γ in autoimmune manifestations observed in this syndrome.

Tfh CELLS AND OTHER INFLAMMATORY AND AUTO-IMMUNE DISEASES

IgA Nephropathy (IgAN) in Adults

IgA nephropathy is the most common form of primary glomerulonephritis in adults, characterized by mesangial deposition of IgG and IgA in glomeruli. Percentages of CD4⁺CXCR5⁺, CD4⁺CXCR5⁺ICOS⁺, and CD4⁺CXCR5⁺PD1⁺ Tfh, as well as a serum level of IL-21, are increased in patients as compared to controls. Percentages of cTfh cells are negatively correlated with renal functioning (eGFR values) while levels of PD1⁺ Tfh cells are positively correlated with levels of galactose-deficient IgA1 (Gd-IgA1, known to be effector molecules in the pathogenesis of IgAN), as well as 24-h urinary proteins. Treatment with prednisone significantly reduces the frequency of cTfh and levels of serum IL-21 (225).

Adults Idiopathic Inflammatory Myopathies (IIM)

Idiopathic inflammatory myopathies are a group of heterogeneous chronic autoimmune diseases comprising dermatomyositis and polymyositis, in which muscles are infiltrated by lymphocytes, dendritic cells, and macrophages. Total cTfh cells are expanded in peripheral blood of patients with IIM compared to controls. This expansion is due to the increase of the Tfh2 and Tfh17 subsets. By contrast, levels of Tfh1 cells are decreased (226). The expansion of Tfh2 and Tfh17 is in line with the work published in 2011 by Morita et al., which was the first study describing cTfh cells and their subsets in humans (56). They described a skewing of cTfh cells subsets toward Tfh2 and Tfh17 phenotypes, in relation to disease activity (skin rash, muscular weakness) and frequency of blood plasmablasts in patients with juvenile dermatomyositis (56).

Systemic Sclerosis (SSc)

Few data are available in literature on the potential role of Tfh cells in SSc pathogenesis. One recent report provides evidence that ICOS⁺ Tfh cells contribute to skin fibrosis (227). T cells with a Tfh phenotype (CD4⁺, CXCR5⁺, and PD-1⁺) and expressing ICOS infiltrate the lesional skin of SSc patients and correlate with dermal fibrosis (assessed by modified Rodnan skin score). Taking advantage of the murine model of sclerodermatous graft-versus-host-disease, authors showed that ICOS⁺ Tfh-like cells contribute to fibrosis *via* IL-21 and matrix metalloproteinase 12. Removal of ICOS⁺ cells ameliorate fibrosis and inhibit dermal inflammation. Finally, by neutralizing specifically IL-21, skin fibrosis is ameliorated as well (227).

CONCLUSION

During past years, significant improvements obtained in Tfh cells biology have led to consider their role in various diseases, such as cancers, immunodeficiencies, allergic diseases, but also in autoimmune disorders. First observations published on mouse models, and specially in lupus-prone ones, have demonstrated the pathogenic role of excessive Tfh cell responses in autoimmunity and have paved the way for subsequent research in humans. Since then, the above-mentioned clinical and experimental findings consistently revealed that patients with autoimmune diseases display aberrant Tfh cells responses, with some common features shared across multiple disorders: increased numbers and proportions of total cTfh cells; alteration in the balance of the subsets, with an increase in activated cTfh2 and cTfh17 cell subsets and a decrease in cTfh1 cells; ectopic GC formation in inflamed tissues; association of Tfh cell numbers and activation state with disease activity, levels of autoantibodies and with the response to conventional treatment. However, unlike mouse studies where lymphoid organs can be easily obtained during disease course, many studies performed to date in human auto-immune diseases have largely investigated Tfh cells only in peripheral blood. Like previously mentioned, the exact origin and biology of these cells remain elusive in humans. Whether the information obtained from the analysis of cTfh cells directly reflects the Tfh responses in SLO and/or in inflamed tissues

remains to be fully established. Data linking human cTfh cell compartment and Tfh cells in lymphoid organs and/or inflamed tissues are still scarce, functional analysis of cTfh cells (ability to deliver B-cell help) is frequently missing in the published studies and exact nature of Tfh cells present in affected organs requires further studies, like the one published recently by Rao et al. in RA (135).

It is also to be determined if the observed alterations in Tfh cells are causative and/or a consequence of the global immune dysregulation present in these diseases, and for instance regarding B cells. It is established that transmission of signals between Tfh cells and GC B cells is bidirectional. Cognate Tfh—B cell interactions are essential for Tfh cells differentiation and maintenance processes, as B cells serve as APCs and as a source of ICOS-L and cytokines (6, 17, 45, 228–232). This interdependent relationship could possibly be harmful in the context of auto-immunity and this feed-forward loop for Tfh cell help delivered by B cells should be considered with precaution. In fact, if expansion of Tfh cells can provide pathological signals of survival and escape to auto-reactive B cells, the reverse statement could also be true. Autoantigens are easily accessible to B cells during auto-immune reactions and this persistent antigenic availability was shown to favor the activation of Tfh cells in a murine model (45). Besides this, in humans, it was observed that after B cell depleting therapy, in lymph nodes, despite the absence of GC, a population of CD4⁺CXCR5⁺CD57⁺ Tfh cells was still present, suggesting that possibly some resident memory Tfh cells do not require B cells for their maintenance (233, 234).

As a whole, the studies mentioned in this review lend support to the existence of a global increase of the activity of the

Tfh lineage in patients with auto-immune and inflammatory disorders and to the more likely association of exaggerated Tfh response with the pathogenesis of human autoimmune diseases. There are still a lot of unanswered and open questions in this field. A better understanding of the biology of these cells, of the events that initiate or sustain their activation is of particular importance, as interference with these processes and their selective inhibition could represent therapeutic options.

AUTHOR CONTRIBUTIONS

CR and NG wrote the first draft of the manuscript. NG, MC, DD, CC-B, M-ET, EL, PD and PB wrote sections of the manuscript. PB and CR revised critically the whole work. All authors contributed to manuscript writing, read and approved the submitted version.

ACKNOWLEDGMENTS

This article is affiliated with the Fédération Hospitalo-Universitaire, Aquitaine's Care, and Research Organization for inflammatory and immune-mediated diseases.

FUNDING

This work was supported by grants from the Société Nationale française de médecine interne (NG) and Société Française de Rhumatologie (PB).

REFERENCES

- Breitfeld D, Ohl L, Kremmer E, Ellwart J, Sallusto F, Lipp M, et al. Follicular B helper T cells express CXC chemokine receptor 5, localize to B cell follicles, and support immunoglobulin production. *J Exp Med* (2000) 192:1545–52. doi:10.1084/jem.192.11.1545
- Campbell DJ, Kim CH, Butcher EC. Separable effector T cell populations specialized for B cell help or tissue inflammation. *Nat Immunol* (2001) 2:876–81. doi:10.1038/ni0901-876
- Kim CH, Rott LS, Clark-Lewis I, Campbell DJ, Wu L, Butcher EC. Subspecialization of CXCR5⁺ T cells: B helper activity is focused in a germinal center-localized subset of CXCR5⁺ T cells. *J Exp Med* (2001) 193:1373–81. doi:10.1084/jem.193.12.1373
- Schaerli P, Willmann K, Lang AB, Lipp M, Loetscher P, Moser B. CXC chemokine receptor 5 expression defines follicular homing T cells with B cell helper function. *J Exp Med* (2000) 192:1553–62. doi:10.1084/jem.192.11.1553
- Arnold CN, Campbell DJ, Lipp M, Butcher EC. The germinal center response is impaired in the absence of T cell-expressed CXCR5. *Eur J Immunol* (2007) 37:100–9. doi:10.1002/eji.200636486
- Haynes NM, Allen CDC, Lesley R, Ansel KM, Killeen N, Cyster JG. Role of CXCR5 and CCR7 in follicular Th cell positioning and appearance of a programmed cell death gene-1^{high} germinal center-associated subpopulation. *J Immunol* (2007) 179:5099–108. doi:10.4049/jimmunol.179.8.5099
- Allen CDC, Okada T, Cyster JG. Germinal-center organization and cellular dynamics. *Immunity* (2007) 27:190–202. doi:10.1016/j.immuni.2007.07.009
- Gunn MD, Ngo VN, Ansel KM, Ekland EH, Cyster JG, Williams LT. A B-cell-homing chemokine made in lymphoid follicles activates Burkitt's lymphoma receptor-1. *Nature* (1998) 391:799–803. doi:10.1038/35876
- Förster R, Mattis AE, Kremmer E, Wolf E, Brem G, Lipp M. A putative chemokine receptor, BLR1, directs B cell migration to defined lymphoid organs and specific anatomic compartments of the spleen. *Cell* (1996) 87:1037–47. doi:10.1016/S0092-8674(00)81798-5
- Ansel KM, McHeyzer-Williams LJ, Ngo VN, McHeyzer-Williams MG, Cyster JG. In vivo-activated CD4 T cells upregulate CXC chemokine receptor 5 and reprogram their response to lymphoid chemokines. *J Exp Med* (1999) 190:1123–34. doi:10.1084/jem.190.8.1123
- Renshaw BR, Fanslow WC, Armitage RJ, Campbell KA, Liggitt D, Wright B, et al. Humoral immune responses in CD40 ligand-deficient mice. *J Exp Med* (1994) 180:1889–900. doi:10.1084/jem.180.5.1889
- Tafuri A, Shahinian A, Bladt F, Yoshinaga SK, Jordana M, Wakeham A, et al. ICOS is essential for effective T-helper-cell responses. *Nature* (2001) 409:105–9. doi:10.1038/35051113
- McAdam AJ, Greenwald RJ, Levin MA, Chernova T, Malenkovich N, Ling V, et al. ICOS is critical for CD40-mediated antibody class switching. *Nature* (2001) 409:102–5. doi:10.1038/35051107
- Dong C, Juedes AE, Temann UA, Shresta S, Allison JP, Ruddle NH, et al. ICOS co-stimulatory receptor is essential for T-cell activation and function. *Nature* (2001) 409:97–101. doi:10.1038/35051100
- Bossaller L, Burger J, Draeger R, Grimbacher B, Knoth R, Plebani A, et al. ICOS deficiency is associated with a severe reduction of CXCR5⁺CD4 germinal center Th cells. *J Immunol* (2006) 177:4927–32. doi:10.4049/jimmunol.177.7.4927
- Akiba H, Takeda K, Kojima Y, Usui Y, Harada N, Yamazaki T, et al. The role of ICOS in the CXCR5⁺ follicular B helper T cell maintenance in vivo. *J Immunol* (2005) 175:2340–8. doi:10.4049/jimmunol.175.4.2340
- Choi YS, Kageyama R, Eto D, Escobar TC, Johnston RJ, Monticelli L, et al. ICOS receptor instructs T follicular helper cell versus effector cell differentiation via induction of the transcriptional repressor Bcl6. *Immunity* (2011) 34:932–46. doi:10.1016/j.immuni.2011.03.023
- Good-Jacobson KL, Szumilas CG, Chen L, Sharpe AH, Tomayko MM, Shlomchik MJ. PD-1 regulates germinal center B cell survival and the formation and affinity of long-lived plasma cells. *Nat Immunol* (2010) 11:535–42. doi:10.1038/ni.1877
- Kim JR, Lim HW, Kang SG, Hillsamer P, Kim CH. Human CD57⁺ germinal center-T cells are the major helpers for GC-B cells and induce class switch recombination. *BMC Immunol* (2005) 6:3. doi:10.1186/1471-2172-6-3

20. Casamayor-Palleja M, Khan M, MacLennan IC. A subset of CD4+ memory T cells contains preformed CD40 ligand that is rapidly but transiently expressed on their surface after activation through the T cell receptor complex. *J Exp Med* (1995) 181:1293–301. doi:10.1084/jem.181.4.1293
21. Kawabe T, Naka T, Yoshida K, Tanaka T, Fujiwara H, Suematsu S, et al. The immune responses in CD40-deficient mice: impaired immunoglobulin class switching and germinal center formation. *Immunity* (1994) 1:167–78. doi:10.1016/1074-7613(94)90095-7
22. Xu J, Foy TM, Laman JD, Elliott EA, Dunn JJ, Waldschmidt TJ, et al. Mice deficient for the CD40 ligand. *Immunity* (1994) 1:423–31. doi:10.1016/1074-7613(94)90073-6
23. Bryant VL, Ma CS, Avery DT, Li Y, Good KL, Corcoran LM, et al. Cytokine-mediated regulation of human B cell differentiation into Ig-secreting cells: predominant role of IL-21 produced by CXCR5+ T follicular helper cells. *J Immunol* (2007) 179:8180–90. doi:10.4049/jimmunol.179.12.8180
24. Yusuf I, Kageyama R, Monticelli L, Johnston RJ, Ditoro D, Hansen K, et al. Germinal center T follicular helper cell IL-4 production is dependent on signaling lymphocytic activation molecule receptor (CD150). *J Immunol* (2010) 185:190–202. doi:10.4049/jimmunol.0903505
25. Reinhardt RL, Liang H-E, Locksley RM. Cytokine-secreting follicular T cells shape the antibody repertoire. *Nat Immunol* (2009) 10:385–93. doi:10.1038/ni.1715
26. King IL, Mohrs M. IL-4-producing CD4+ T cells in reactive lymph nodes during helminth infection are T follicular helper cells. *J Exp Med* (2009) 206:1001–7. doi:10.1084/jem.20090313
27. Ettinger R, Sims GP, Fairhurst A-M, Robbins R, da Silva YS, Spolski R, et al. IL-21 induces differentiation of human naive and memory B cells into antibody-secreting plasma cells. *J Immunol* (2005) 175:7867–79. doi:10.4049/jimmunol.175.12.7867
28. Pène J, Gauchat J-F, Lécart S, Drouet E, Guglielmi P, Boulay V, et al. Cutting edge: IL-21 is a switch factor for the production of IgG₁ and IgG₂ by human B cells. *J Immunol* (2004) 172:5154–7. doi:10.4049/jimmunol.172.9.5154
29. Ozaki K, Spolski R, Feng CG, Qi C-F, Cheng J, Sher A, et al. A critical role for IL-21 in regulating immunoglobulin production. *Science* (2002) 298:1630–4. doi:10.1126/science.1077002
30. Kuchen S, Robbins R, Sims GP, Sheng C, Phillips TM, Lipsky PE, et al. Essential role of IL-21 in B cell activation, expansion, and plasma cell generation during CD4+ T cell-B cell collaboration. *J Immunol* (2007) 179:5886–96. doi:10.4049/jimmunol.179.9.5886
31. Suto A, Kashiwakuma D, Kagami S, Hirose K, Watanabe N, Yokote K, et al. Development and characterization of IL-21-producing CD4+ T cells. *J Exp Med* (2008) 205:1369–79. doi:10.1084/jem.20072057
32. Caprioli F, Sarra M, Caruso R, Stolfi C, Fina D, Sica G, et al. Autocrine regulation of IL-21 production in human T lymphocytes. *J Immunol* (2008) 180:1800–7. doi:10.4049/jimmunol.180.3.1800
33. Nurieva R, Yang XO, Martinez G, Zhang Y, Panopoulos AD, Ma L, et al. Essential autocrine regulation by IL-21 in the generation of inflammatory T cells. *Nature* (2007) 448:480–3. doi:10.1038/nature05969
34. Nurieva RI, Chung Y, Hwang D, Yang XO, Kang HS, Ma L, et al. Generation of T follicular helper cells is mediated by interleukin-21 but independent of T helper 1, 2, or 17 cell lineages. *Immunity* (2008) 29:138–49. doi:10.1016/j.immuni.2008.05.009
35. Vogelzang A, McGuire HM, Yu D, Sprent J, Mackay CR, King C. A fundamental role for interleukin-21 in the generation of T follicular helper cells. *Immunity* (2008) 29:127–37. doi:10.1016/j.immuni.2008.06.001
36. Yu D, Rao S, Tsai LM, Lee SK, He Y, Sutcliffe EL, et al. The transcriptional repressor Bcl-6 directs T follicular helper cell lineage commitment. *Immunity* (2009) 31:457–68. doi:10.1016/j.immuni.2009.07.002
37. Nurieva RI, Chung Y, Martinez GJ, Yang XO, Tanaka S, Matskevitch TD, et al. Bcl6 mediates the development of T follicular helper cells. *Science* (2009) 325:1001–5. doi:10.1126/science.1176676
38. Johnston RJ, Poholek AC, DiToro D, Yusuf I, Eto D, Barnett B, et al. Bcl6 and Blimp-1 are reciprocal and antagonistic regulators of T follicular helper cell differentiation. *Science* (2009) 325:1006–10. doi:10.1126/science.1175870
39. Betz BC, Jordan-Williams KL, Wang C, Kang SG, Liao J, Logan MR, et al. Batf coordinates multiple aspects of B and T cell function required for normal antibody responses. *J Exp Med* (2010) 207:933–42. doi:10.1084/jem.20091548
40. Liu X, Chen X, Zhong B, Wang A, Wang X, Chu F, et al. Transcription factor achaete-scute homologue 2 initiates follicular T-helper-cell development. *Nature* (2014) 507:513–8. doi:10.1038/nature12910
41. Bollig N, Brüstle A, Kellner K, Ackermann W, Abass E, Raifer H, et al. Transcription factor IRF4 determines germinal center formation through follicular T-helper cell differentiation. *Proc Natl Acad Sci U S A* (2012) 109:8664–9. doi:10.1073/pnas.1205834109
42. Kitano M, Moriyama S, Ando Y, Hikida M, Mori Y, Kurosaki T, et al. Bcl6 protein expression shapes pre-germinal center B cell dynamics and follicular helper T cell heterogeneity. *Immunity* (2011) 34:961–72. doi:10.1016/j.immuni.2011.03.025
43. Baumjohann D, Okada T, Ansel KM. Cutting edge: distinct waves of BCL6 expression during T follicular helper cell development. *J Immunol* (2011) 187:2089–92. doi:10.4049/jimmunol.1101393
44. Deenick EK, Chan A, Ma CS, Gatto D, Schwartzberg PL, Brink R, et al. Follicular helper T cell differentiation requires continuous antigen presentation that is independent of unique B cell signaling. *Immunity* (2010) 33:241–53. doi:10.1016/j.immuni.2010.07.015
45. Baumjohann D, Preite S, Reboldi A, Ronchi F, Ansel KM, Lanzavecchia A, et al. Persistent antigen and germinal center B cells sustain T follicular helper cell responses and phenotype. *Immunity* (2013) 38:596–605. doi:10.1016/j.immuni.2012.11.020
46. Goenka R, Barnett LG, Silver JS, O'Neill PJ, Hunter CA, Cancro MP, et al. Cutting edge: dendritic cell-restricted antigen presentation initiates the follicular helper T cell program but cannot complete ultimate effector differentiation. *J Immunol* (2011) 187:1091–5. doi:10.4049/jimmunol.1100853
47. Schmitt N, Morita R, Bourdery L, Bentebibel SE, Zurawski SM, Banchereau J, et al. Human dendritic cells induce the differentiation of interleukin-21-producing T follicular helper-like cells through interleukin-12. *Immunity* (2009) 31:158–69. doi:10.1016/j.immuni.2009.04.016
48. Schmitt N, Bustamante J, Bourdery L, Bentebibel SE, Boisson-Dupuis S, Hamlin F, et al. IL-12 receptor β 1 deficiency alters in vivo T follicular helper cell response in humans. *Blood* (2013) 121:3375–85. doi:10.1182/blood-2012-08-448902
49. Ma CS, Suryani S, Avery DT, Chan A, Nanan R, Santner-Nanan B, et al. Early commitment of naïve human CD4(+) T cells to the T follicular helper (TFH) cell lineage is induced by IL-12. *Immunol Cell Biol* (2009) 87:590–600. doi:10.1038/icb.2009.64
50. Schmitt N, Liu Y, Bentebibel S-E, Munagala I, Bourdery L, Venuprasad K, et al. The cytokine TGF- β co-opts signaling via STAT3-STAT4 to promote the differentiation of human TFH cells. *Nat Immunol* (2014) 15:856–65. doi:10.1038/ni.2947
51. Ballesteros-Tato A, León B, Graf BA, Moquin A, Adams PS, Lund FE, et al. Interleukin-2 inhibits germinal center formation by limiting T follicular helper cell differentiation. *Immunity* (2012) 36:847–56. doi:10.1016/j.immuni.2012.02.012
52. León B, Bradley JE, Lund FE, Randall TD, Ballesteros-Tato A. FoxP3+ regulatory T cells promote influenza-specific Tfh responses by controlling IL-2 availability. *Nat Commun* (2014) 5:3495. doi:10.1038/ncomms4495
53. Johnston RJ, Choi YS, Diamond JA, Yang JA, Crotty S. STAT5 is a potent negative regulator of TFH cell differentiation. *J Exp Med* (2012) 209:243–50. doi:10.1084/jem.20111174
54. Tsai LM, Yu D. Follicular helper T-cell memory: establishing new frontiers during antibody response. *Immunol Cell Biol* (2014) 92:57–63. doi:10.1038/icb.2013.68
55. Trüb M, Barr TA, Morrison VL, Brown S, Caserta S, Rixon J, et al. Heterogeneity of phenotype and function reflects the multistage development of T follicular helper cells. *Front Immunol* (2017) 8:489. doi:10.3389/fimmu.2017.00489
56. Morita R, Schmitt N, Bentebibel S-E, Ranganathan R, Bourdery L, Zurawski G, et al. Human blood CXCR5(+)CD4(+) T cells are counterparts of T follicular cells and contain specific subsets that differentially support antibody secretion. *Immunity* (2011) 34:108–21. doi:10.1016/j.immuni.2010.12.012
57. Bentebibel S-E, Lopez S, Obermoser G, Schmitt N, Mueller C, Harrod C, et al. Induction of ICOS+CXCR3+CXCR5+ TH cells correlates with antibody responses to influenza vaccination. *Sci Transl Med* (2013) 5:176ra32. doi:10.1126/scitranslmed.3005191
58. He J, Tsai LM, Leong YA, Hu X, Ma CS, Chevalier N, et al. Circulating precursor CCR7(lo)PD-1(hi) CXCR5+ CD4+ T cells indicate Tfh cell activity and promote antibody responses upon antigen reexposure. *Immunity* (2013) 39:770–81. doi:10.1016/j.immuni.2013.09.007

59. Schmitt N, Bentebibel S-E, Ueno H. Phenotype and functions of memory Tfh cells in human blood. *Trends Immunol* (2014) 35:436–42. doi:10.1016/j.it.2014.06.002
60. Locci M, Havenar-Daughton C, Landais E, Wu J, Kroenke MA, Arlehamn CL, et al. Human circulating PD-1+CXCR3-CXCR5+ memory Tfh cells are highly functional and correlate with broadly neutralizing HIV antibody responses. *Immunity* (2013) 39:758–69. doi:10.1016/j.immuni.2013.08.031
61. Schmitt N, Ueno H. Blood Tfh cells come with colors. *Immunity* (2013) 39:629–30. doi:10.1016/j.immuni.2013.09.011
62. Wei Y, Feng J, Hou Z, Wang XM, Yu D. Flow cytometric analysis of circulating follicular helper T (Tfh) and follicular regulatory T (Tfr) populations in human blood. *Methods Mol Biol* (2015) 1291:199–207. doi:10.1007/978-1-4939-2498-1_17
63. Alexander C-M, Tygrett LT, Boyden AW, Wolniak KL, Legge KL, Waldschmidt TJ. T regulatory cells participate in the control of germinal centre reactions. *Immunology* (2011) 133:452–68. doi:10.1111/j.1365-2567.2011.03456.x
64. Chung Y, Tanaka S, Chu F, Nurieva RI, Martinez GJ, Rawal S, et al. Follicular regulatory T cells expressing Foxp3 and Bcl-6 suppress germinal center reactions. *Nat Med* (2011) 17:983–8. doi:10.1038/nm.2426
65. Linterman MA, Pierson W, Lee SK, Kallies A, Kawamoto S, Rayner TF, et al. Foxp3+ follicular regulatory T cells control the germinal center response. *Nat Med* (2011) 17:975–82. doi:10.1038/nm.2425
66. Wollenberg I, Agua-Doce A, Hernández A, Almeida C, Oliveira VG, Faro J, et al. Regulation of the germinal center reaction by Foxp3+ follicular regulatory T cells. *J Immunol* (2011) 187:4553–60. doi:10.4049/jimmunol.1101328
67. Corsiero E, Nerviani A, Bombardieri M, Pitzalis C. Ectopic lymphoid structures: powerhouse of autoimmunity. *Front Immunol* (2016) 7:430. doi:10.3389/fimmu.2016.00430
68. Vu Van D, Beier KC, Pietzke L-J, Al Baz MS, Feist RK, Gurka S, et al. Local T/B cooperation in inflamed tissues is supported by T follicular helper-like cells. *Nat Commun* (2016) 7:10875. doi:10.1038/ncomms10875
69. Lisnevskaja L, Murphy G, Isenberg D. Systemic lupus erythematosus. *Lancet Lond Engl* (2014) 384:1878–88. doi:10.1016/S0140-6736(14)60128-8
70. Hostmann A, Jacobi AM, Mei H, Hiepe F, Dörner T. Peripheral B cell abnormalities and disease activity in systemic lupus erythematosus. *Lupus* (2008) 17:1064–9. doi:10.1177/0961203308095138
71. Kim YU, Lim H, Jung HE, Wetsel RA, Chung Y. Regulation of autoimmune germinal center reactions in lupus-prone BXD2 mice by follicular helper T cells. *PLoS One* (2015) 10:e0120294. doi:10.1371/journal.pone.0120294
72. Vinuesa CG, Cook MC, Angelucci C, Athanasopoulos V, Rui L, Hill KM, et al. A RING-type ubiquitin ligase family member required to repress follicular helper T cells and autoimmunity. *Nature* (2005) 435:452–8. doi:10.1038/nature03555
73. Vogel KU, Edelmann SL, Jeltsch KM, Bertossi A, Heger K, Heinz GA, et al. Roquin paralogs 1 and 2 redundantly repress the Icos and OX40 costimulator mRNAs and control follicular helper T cell differentiation. *Immunity* (2013) 38:655–68. doi:10.1016/j.immuni.2012.12.004
74. Pratama A, Ramiscal RR, Silva DG, Das SK, Athanasopoulos V, Fitch J, et al. Roquin-2 shares functions with its paralog Roquin-1 in the repression of mRNAs controlling T follicular helper cells and systemic inflammation. *Immunity* (2013) 38:669–80. doi:10.1016/j.immuni.2013.01.011
75. Linterman MA, Rigby RJ, Wong RK, Yu D, Brink R, Cannons JL, et al. Follicular helper T cells are required for systemic autoimmunity. *J Exp Med* (2009) 206:561–76. doi:10.1084/jem.20081886
76. Murphy ED, Roths JB. A Y chromosome associated factor in strain BXSB producing accelerated autoimmunity and lymphoproliferation. *Arthritis Rheum* (1979) 22:1188–94. doi:10.1002/art.1780221105
77. Bubier JA, Sproule TJ, Foreman O, Spolski R, Shaffer DJ, Morse HC, et al. A critical role for IL-21 receptor signaling in the pathogenesis of systemic lupus erythematosus in BXSB-Yaa mice. *Proc Natl Acad Sci U S A* (2009) 106:1518–23. doi:10.1073/pnas.0807309106
78. Ozaki K, Spolski R, Ettinger R, Kim H-P, Wang G, Qi C-F, et al. Regulation of B cell differentiation and plasma cell generation by IL-21, a novel inducer of Blimp-1 and Bcl-6. *J Immunol* (2004) 173:5361–71. doi:10.4049/jimmunol.173.9.5361
79. Bubier JA, Bennett SM, Sproule TJ, Lyons BL, Olland S, Young DA, et al. Treatment of BXSB-Yaa mice with IL-21R-Fc fusion protein minimally attenuates systemic lupus erythematosus. *Ann N Y Acad Sci* (2007) 1110:590–601. doi:10.1196/annals.1423.063
80. McPhee CG, Bubier JA, Sproule TJ, Park G, Steinbuck MP, Schott WH, et al. IL-21 is a double-edged sword in the systemic lupus erythematosus-like disease of BXSB-Yaa mice. *J Immunol* (2013) 191:4581–8. doi:10.4049/jimmunol.1300439
81. Rankin AL, Guay H, Herber D, Bertino SA, Duzanski TA, Carrier Y, et al. IL-21 receptor is required for the systemic accumulation of activated B and T lymphocytes in MRL/MpJ-Faslpr/lpr/J mice. *J Immunol* (2012) 188:1656–67. doi:10.4049/jimmunol.1003871
82. Herber D, Brown TP, Liang S, Young DA, Collins M, Dunussi-Joannopoulos K. IL-21 has a pathogenic role in a lupus-prone mouse model and its blockade with IL-21R.Fc reduces disease progression. *J Immunol* (2007) 178:3822–30. doi:10.4049/jimmunol.178.6.3822
83. Zhang M, Yu G, Chan B, Pearson JT, Rathanaswami P, Delaney J, et al. Interleukin-21 receptor blockade inhibits secondary humoral responses and halts the progression of preestablished disease in the (NZB × NZW)F1 systemic lupus erythematosus model. *Arthritis Rheumatol* (2015) 67:2723–31. doi:10.1002/art.39233
84. Hu Y-L, Metz DP, Chung J, Siu G, Zhang M. B7RP-1 blockade ameliorates autoimmunity through regulation of follicular helper T cells. *J Immunol* (2009) 182:1421–8. doi:10.4049/jimmunol.182.3.1421
85. Iwai H, Abe M, Hirose S, Tsushima F, Tezuka K, Akiba H, et al. Involvement of inducible costimulator-B7 homologous protein costimulatory pathway in murine lupus nephritis. *J Immunol* (2003) 171:2848–54. doi:10.4049/jimmunol.171.6.2848
86. Zeller GC, Hirahashi J, Schwarting A, Sharpe AH, Kelley VR. Inducible co-stimulator null MRL-Faslpr mice: uncoupling of autoantibodies and T cell responses in lupus. *J Am Soc Nephrol* (2006) 17:122–30. doi:10.1681/ASN.2005080802
87. Mittereder N, Kuta E, Bhat G, Dacosta K, Cheng LI, Herbst R, et al. Loss of immune tolerance is controlled by ICOS in Sle1 mice. *J Immunol* (2016) 197:491–503. doi:10.4049/jimmunol.1502241
88. Simpson N, Gatenby PA, Wilson A, Malik S, Fulcher DA, Tangye SG, et al. Expansion of circulating T cells resembling follicular helper T cells is a fixed phenotype that identifies a subset of severe systemic lupus erythematosus. *Arthritis Rheum* (2010) 62:234–44. doi:10.1002/art.25032
89. Terrier B, Costedoat-Chalumeau N, Garrido M, Geri G, Rosenzweig M, Musset L, et al. Interleukin 21 correlates with T cell and B cell subset alterations in systemic lupus erythematosus. *J Rheumatol* (2012) 39:1819–28. doi:10.3899/jrheum.120468
90. Feng X, Wang D, Chen J, Lu L, Hua B, Li X, et al. Inhibition of aberrant circulating Th cell proportions by corticosteroids in patients with systemic lupus erythematosus. *PLoS One* (2012) 7:e51982. doi:10.1371/journal.pone.0051982
91. Wang L, Zhao P, Ma L, Shan Y, Jiang Z, Wang J, et al. Increased interleukin 21 and follicular helper T-like cells and reduced interleukin 10+ B cells in patients with new-onset systemic lupus erythematosus. *J Rheumatol* (2014) 41:1781–92. doi:10.3899/jrheum.131025
92. Yang X, Yang J, Chu Y, Xue Y, Xuan D, Zheng S, et al. T follicular helper cells and regulatory B cells dynamics in systemic lupus erythematosus. *PLoS One* (2014) 9:e88441. doi:10.1371/journal.pone.0088441
93. Zhang X, Lindwall E, Gauthier C, Lyman J, Spencer N, Alarakhia A, et al. Circulating CXCR5+CD4+helper T cells in systemic lupus erythematosus patients share phenotypic properties with germinal center follicular helper T cells and promote antibody production. *Lupus* (2015) 24:909–17. doi:10.1177/0961203314567750
94. Choi J-Y, Ho JH, Pasoto SG, Bunin V, Kim ST, Carrasco S, et al. Circulating follicular helper-like T cells in systemic lupus erythematosus: association with disease activity. *Arthritis Rheumatol* (2015) 67:988–99. doi:10.1002/art.39020
95. Szabó K, Papp G, Szántó A, Tarr T, Zeher M. A comprehensive investigation on the distribution of circulating follicular T helper cells and B cell subsets in primary Sjögren's syndrome and systemic lupus erythematosus. *Clin Exp Immunol* (2016) 183:76–89. doi:10.1111/cei.12703
96. Umeda M, Koga T, Ichinose K, Igawa T, Sato T, Takatani A, et al. CD4+CD52loT-cell expression contributes to the development of systemic lupus erythematosus. *Clin Immunol* (2018) 187:50–7. doi:10.1016/j.clim.2017.10.004

97. Xu H, Liu J, Cui X, Zuo Y, Zhang Z, Li Y, et al. Increased frequency of circulating follicular helper T cells in lupus patients is associated with auto-antibody production in a CD40L-dependent manner. *Cell Immunol* (2015) 295:46–51. doi:10.1016/j.cellimm.2015.01.014
98. Huang X, Wu H, Qiu H, Yang H, Deng Y, Zhao M, et al. The expression of Bcl-6 in circulating follicular helper-like T cells positively correlates with the disease activity in systemic lupus erythematosus. *Clin Immunol* (2016) 173:161–70. doi:10.1016/j.clim.2016.10.017
99. Le Coz C, Joubin A, Pasquali J-L, Korganow A-S, Dumortier H, Monneaux F. Circulating TFH subset distribution is strongly affected in lupus patients with an active disease. *PLoS One* (2013) 8:e75319. doi:10.1371/journal.pone.0075319
100. Lan Y, Luo B, Wang J-L, Jiang Y-W, Wei Y-S. The association of interleukin-21 polymorphisms with interleukin-21 serum levels and risk of systemic lupus erythematosus. *Gene* (2014) 538:94–8. doi:10.1016/j.gene.2014.01.012
101. Wong CK, Wong PTY, Tam LS, Li EK, Chen DP, Lam CWK. Elevated production of B cell chemokine CXCL13 is correlated with systemic lupus erythematosus disease activity. *J Clin Immunol* (2010) 30:45–52. doi:10.1007/s10875-009-9325-5
102. Lee J, Shin E-K, Lee S-Y, Her Y-M, Park M-K, Kwok S-K, et al. Oestrogen up-regulates interleukin-21 production by CD4(+) T lymphocytes in patients with systemic lupus erythematosus. *Immunology* (2014) 142:573–80. doi:10.1111/imm.12263
103. Nakou M, Papadimitraki ED, Fanouriakis A, Bertsias GK, Choulaki C, Goulidaki N, et al. Interleukin-21 is increased in active systemic lupus erythematosus patients and contributes to the generation of plasma B cells. *Clin Exp Rheumatol* (2013) 31:172–9.
104. Doff S, Abdulahad WH, Westra J, Doornbos-van der Meer B, Limburg PC, Kallenberg CGM, et al. Increase in IL-21 producing T-cells in patients with systemic lupus erythematosus. *Arthritis Res Ther* (2011) 13:R157. doi:10.1186/ar3474
105. Liarski VM, Kaverina N, Chang A, Brandt D, Yanez D, Talasnik L, et al. Cell distance mapping identifies functional T follicular helper cells in inflamed human renal tissue. *Sci Transl Med* (2014) 6:230ra46. doi:10.1126/scitranslmed.3008146
106. Chang A, Henderson SG, Brandt D, Liu N, Guttikonda R, Hsieh C, et al. In situ B cell-mediated immune responses and tubulointerstitial inflammation in human lupus nephritis. *J Immunol* (2011) 186:1849–60. doi:10.4049/jimmunol.1001983
107. Xu B, Wang S, Zhou M, Huang Y, Fu R, Guo C, et al. The ratio of circulating follicular T helper cell to follicular T regulatory cell is correlated with disease activity in systemic lupus erythematosus. *Clin Immunol* (2017) 183:46–53. doi:10.1016/j.clim.2017.07.004
108. Ma L, Zhao P, Jiang Z, Shan Y, Jiang Y. Imbalance of different types of CD4(+) forkhead box protein 3 (FoxP3)(+) T cells in patients with new-onset systemic lupus erythematosus. *Clin Exp Immunol* (2013) 174:345–55. doi:10.1111/cei.12189
109. Liu C, Wang D, Song Y, Lu S, Zhao J, Wang H. Increased circulating CD4+CXCR5+FoxP3+follicular regulatory T cells correlated with severity of systemic lupus erythematosus patients. *Int Immunopharmacol* (2018) 56:261–8. doi:10.1016/j.intimp.2018.01.038
110. He J, Zhang X, Wei Y, Sun X, Chen Y, Deng J, et al. Low-dose interleukin-2 treatment selectively modulates CD4(+) T cell subsets in patients with systemic lupus erythematosus. *Nat Med* (2016) 22:991–3. doi:10.1038/nm.4148
111. Jacquemin C, Schmitt N, Contain-Bordes C, Liu Y, Narayanan P, Seneschal J, et al. OX40 ligand contributes to human lupus pathogenesis by promoting T follicular helper response. *Immunity* (2015) 42:1159–70. doi:10.1016/j.immuni.2015.05.012
112. Ueno H, Blanco P. OX40/OX40L axis: not a friend in autoimmunity. *Oncotarget* (2015) 6:21779–80. doi:10.18632/oncotarget.4973
113. Smolen JS, Aletaha D, McInnes IB. Rheumatoid arthritis. *Lancet Lond Engl* (2016) 388:2023–38. doi:10.1016/S0140-6736(16)30173-8
114. Moschovakis GL, Bubke A, Friedrichsen M, Falk CS, Feederle R, Förster R. T cell specific Cxcr5 deficiency prevents rheumatoid arthritis. *Sci Rep* (2017) 7:8933. doi:10.1038/s41598-017-08935-6
115. Chevalier N, Macia L, Tan JK, Mason LJ, Robert R, Thorburn AN, et al. The role of follicular helper T cell molecules and environmental influences in autoantibody production and progression to inflammatory arthritis in mice. *Arthritis Rheumatol* (2016) 68:1026–38. doi:10.1002/art.39481
116. Arroyo-Villa I, Bautista-Caro M-B, Balsa A, Aguado-Acín P, Bonilla-Hernán M-G, Plasencia C, et al. Constitutively altered frequencies of circulating follicular helper T cell counterparts and their subsets in rheumatoid arthritis. *Arthritis Res Ther* (2014) 16:500. doi:10.1186/s13075-014-0500-6
117. Zhang Y, Li Y, Lv T-T, Yin Z-J, Wang X-B. Elevated circulating Th17 and follicular helper CD4(+) T cells in patients with rheumatoid arthritis. *APMIS* (2015) 123:659–66. doi:10.1111/apm.12399
118. Nakayama S, Kubo S, Yoshikawa M, Miyazaki Y, Yunoue N, Iwata S, et al. Differential effects of biological DMARDs on peripheral immune cell phenotypes in patients with rheumatoid arthritis. *Rheumatology (Oxford)* (2018) 57:164–74. doi:10.1093/rheumatology/kex012
119. Wang J, Shan Y, Jiang Z, Feng J, Li C, Ma L, et al. High frequencies of activated B cells and T follicular helper cells are correlated with disease activity in patients with new-onset rheumatoid arthritis. *Clin Exp Immunol* (2013) 174:212–20. doi:10.1111/cei.12162
120. Ma J, Zhu C, Ma B, Tian J, Baidoo SE, Mao C, et al. Increased frequency of circulating follicular helper T cells in patients with rheumatoid arthritis. *Clin Dev Immunol* (2012) 2012:827480. doi:10.1155/2012/827480
121. Liu R, Wu Q, Su D, Che N, Chen H, Geng L, et al. A regulatory effect of IL-21 on T follicular helper-like cell and B cell in rheumatoid arthritis. *Arthritis Res Ther* (2012) 14:R255. doi:10.1186/ar4100
122. Penatti A, Facciotti F, De Matteis R, Larghi P, Paroni M, Murgo A, et al. Differences in serum and synovial CD4+ T cells and cytokine profiles to stratify patients with inflammatory osteoarthritis and rheumatoid arthritis. *Arthritis Res Ther* (2017) 19:103. doi:10.1186/s13075-017-1305-1
123. Chu Y, Wang F, Zhou M, Chen L, Lu Y. A preliminary study on the characterization of follicular helper T (Tfh) cells in rheumatoid arthritis synovium. *Acta Histochem* (2014) 116:539–43. doi:10.1016/j.acthis.2013.10.009
124. Costantino AB, Acosta CDV, Onetti L, Mussano E, Cadile II, Ferrero PV. Follicular helper T cells in peripheral blood of patients with rheumatoid arthritis. *Reumatol Clin* (2017) 13:338–43. doi:10.1016/j.reuma.2016.07.003
125. Chakera A, Bennett SC, Morteau O, Bowness P, Luqmani RA, Cornall RJ. The phenotype of circulating follicular-helper T cells in patients with rheumatoid arthritis defines CD200 as a potential therapeutic target. *Clin Dev Immunol* (2012) 2012:948218. doi:10.1155/2012/948218
126. Jüngel A, Distler JHW, Kurowska-Stolarska M, Seemayer CA, Seibl R, Forster A, et al. Expression of interleukin-21 receptor, but not interleukin-21, in synovial fibroblasts and synovial macrophages of patients with rheumatoid arthritis. *Arthritis Rheum* (2004) 50:1468–76. doi:10.1002/art.20218
127. Li J, Shen W, Kong K, Liu Z. Interleukin-21 induces T-cell activation and proinflammatory cytokine secretion in rheumatoid arthritis. *Scand J Immunol* (2006) 64:515–22. doi:10.1111/j.1365-3083.2006.01795.x
128. Xing R, Jin Y, Sun L, Yang L, Li C, Li Z, et al. Interleukin-21 induces migration and invasion of fibroblast-like synoviocytes from patients with rheumatoid arthritis. *Clin Exp Immunol* (2016) 184:147–58. doi:10.1111/cei.12751
129. Andersson AK, Feldmann M, Brennan FM. Neutralizing IL-21 and IL-15 inhibits pro-inflammatory cytokine production in rheumatoid arthritis. *Scand J Immunol* (2008) 68:103–11. doi:10.1111/j.1365-3083.2008.02118.x
130. Jang E, Cho S-H, Park H, Paik D-J, Kim JM, Youn J. A positive feedback loop of IL-21 signaling provoked by homeostatic CD4+CD25- T cell expansion is essential for the development of arthritis in autoimmune K/BxN mice. *J Immunol* (2009) 182:4649–56. doi:10.4049/jimmunol.0804350
131. Platt AM, Gibson VB, Patakas A, Benson RA, Nadler SG, Brewer JM, et al. Abatacept limits breach of self-tolerance in a murine model of arthritis via effects on the generation of T follicular helper cells. *J Immunol* (2010) 185:1558–67. doi:10.4049/jimmunol.1001311
132. Teng F, Klinger CN, Felix KM, Bradley CP, Wu E, Tran NL, et al. Gut microbiota drive autoimmune arthritis by promoting differentiation and migration of Peyer's Patch T follicular helper cells. *Immunity* (2016) 44:875–88. doi:10.1016/j.immuni.2016.03.013
133. Wu H-J, Ivanov II, Darce J, Hattori K, Shima T, Umesaki Y, et al. Gut-residing segmented filamentous bacteria drive autoimmune arthritis via T helper 17 cells. *Immunity* (2010) 32:815–27. doi:10.1016/j.immuni.2010.06.001
134. Block KE, Zheng Z, Dent AL, Kee BL, Huang H. Gut microbiota regulates K/BxN autoimmune arthritis through follicular helper T but not Th17 cells. *J Immunol* (2016) 196:1550–7. doi:10.4049/jimmunol.1501904

135. Rao DA, Gurish MF, Marshall JL, Slowikowski K, Fonseka CY, Liu Y, et al. Pathologically expanded peripheral T helper cell subset drives B cells in rheumatoid arthritis. *Nature* (2017) 542:110–4. doi:10.1038/nature20810
136. Xiao F, Zhang H-Y, Liu Y-J, Zhao D, Shan Y-X, Jiang Y-F. Higher frequency of peripheral blood interleukin 21 positive follicular helper T cells in patients with ankylosing spondylitis. *J Rheumatol* (2013) 40:2029–37. doi:10.3899/jrheum.130125
137. Shan Y, Qi C, Zhao J, Liu Y, Gao H, Zhao D, et al. Higher frequency of peripheral blood follicular regulatory T cells in patients with new onset ankylosing spondylitis. *Clin Exp Pharmacol Physiol* (2015) 42:154–61. doi:10.1111/1440-1681.12330
138. Bautista-Caro M-B, Arroyo-Villa I, Castillo-Gallego C, de Miguel E, Peiteado D, Plasencia-Rodríguez C, et al. Decreased frequencies of circulating follicular helper T cell counterparts and plasmablasts in ankylosing spondylitis patients Naïve for TNF blockers. *PLoS One* (2014) 9:e107086. doi:10.1371/journal.pone.0107086
139. Fox RI. Sjögren's syndrome. *Lancet Lond Engl* (2005) 366:321–31. doi:10.1016/S0140-6736(05)66990-5
140. Nocturne G, Mariette X. B cells in the pathogenesis of primary Sjögren syndrome. *Nat Rev Rheumatol* (2018) 14:133–45. doi:10.1038/nrrheum.2018.1
141. Jin L, Yu D, Li X, Yu N, Li X, Wang Y, et al. CD4+CXCR5+ follicular helper T cells in salivary gland promote B cells maturation in patients with primary Sjögren's syndrome. *Int J Clin Exp Pathol* (2014) 7:1988–96.
142. Li X, Wu Z, Ding J, Zheng Z, Li X, Chen L, et al. Role of the frequency of blood CD4(+) CXCR5(+) CCR6(+) T cells in autoimmunity in patients with Sjögren's syndrome. *Biochem Biophys Res Commun* (2012) 422:238–44. doi:10.1016/j.bbrc.2012.04.133
143. Szabo K, Papp G, Barath S, Gyimesi E, Szanto A, Zeher M. Follicular helper T cells may play an important role in the severity of primary Sjögren's syndrome. *Clin Immunol* (2013) 147:95–104. doi:10.1016/j.clim.2013.02.024
144. Kang KY, Kim H-O, Kwok S-K, Ju JH, Park K-S, Sun D-I, et al. Impact of interleukin-21 in the pathogenesis of primary Sjögren's syndrome: increased serum levels of interleukin-21 and its expression in the labial salivary glands. *Arthritis Res Ther* (2011) 13:R179. doi:10.1186/ar3504
145. Gong Y-Z, Nititham J, Taylor K, Miceli-Richard C, Sordet C, Wachsmann D, et al. Differentiation of follicular helper T cells by salivary gland epithelial cells in primary Sjögren's syndrome. *J Autoimmun* (2014) 51:57–66. doi:10.1016/j.jaut.2013.11.003
146. Maehara T, Moriyama M, Hayashida J-N, Tanaka A, Shinozaki S, Kubo Y, et al. Selective localization of T helper subsets in labial salivary glands from primary Sjögren's syndrome patients. *Clin Exp Immunol* (2012) 169:89–99. doi:10.1111/j.1365-2249.2012.04606.x
147. Verstappen GM, Kroese FGM, Meiners PM, Corneth OB, Huitema MG, Haacke EA, et al. B cell depletion therapy normalizes circulating follicular Th cells in primary Sjögren syndrome. *J Rheumatol* (2017) 44:49–58. doi:10.3899/jrheum.160313
148. Verstappen GM, Meiners PM, Corneth OBJ, Visser A, Arends S, Abdulahad WH, et al. Attenuation of follicular helper T cell-dependent B cell hyperactivity by abatacept treatment in primary Sjögren's syndrome. *Arthritis Rheumatol* (2017) 69:1850–61. doi:10.1002/art.40165
149. Reich DS, Lucchinetti CF, Calabresi PA. Multiple sclerosis. *N Engl J Med* (2018) 378:169–80. doi:10.1056/NEJMra1401483
150. Magliozzi R, Howell O, Vora A, Serafini B, Nicholas R, Puopolo M, et al. Meningeal B-cell follicles in secondary progressive multiple sclerosis associate with early onset of disease and severe cortical pathology. *Brain* (2007) 130:1089–104. doi:10.1093/brain/awm038
151. Howell OW, Reeves CA, Nicholas R, Carassiti D, Radotra B, Gentleman SM, et al. Meningeal inflammation is widespread and linked to cortical pathology in multiple sclerosis. *Brain* (2011) 134:2755–71. doi:10.1093/brain/awr182
152. Romme Christensen J, Börnsen L, Ratzer R, Piehl F, Khademi M, Olsson T, et al. Systemic inflammation in progressive multiple sclerosis involves follicular T-helper, Th17- and activated B-cells and correlates with progression. *PLoS One* (2013) 8:e57820. doi:10.1371/journal.pone.0057820
153. Fan X, Jin T, Zhao S, Liu C, Han J, Jiang X, et al. Circulating CCR7+ICOS+ memory T follicular helper cells in patients with multiple sclerosis. *PLoS One* (2015) 10:e0134523. doi:10.1371/journal.pone.0134523
154. Wingerchuk DM, Lennon VA, Lucchinetti CF, Pittock SJ, Weinshenker BG. The spectrum of neuromyelitis optica. *Lancet Neurol* (2007) 6:805–15. doi:10.1016/S1474-4422(07)70216-8
155. Li Y-J, Zhang F, Qi Y, Chang G-Q, Fu Y, Su L, et al. Association of circulating follicular helper T cells with disease course of NMO spectrum disorders. *J Neuroimmunol* (2015) 278:239–46. doi:10.1016/j.jneuroim.2014.11.011
156. Fan X, Jiang Y, Han J, Liu J, Wei Y, Jiang X, et al. Circulating memory T follicular helper cells in patients with neuromyelitis optica/neuromyelitis optica spectrum disorders. *Mediators Inflamm* (2016) 2016:3678152. doi:10.1155/2016/3678152
157. Gilhus NE. Myasthenia gravis. *N Engl J Med* (2016) 375:2570–81. doi:10.1056/NEJMra1602678
158. Saito R, Onodera H, Tago H, Suzuki Y, Shimizu M, Matsumura Y, et al. Altered expression of chemokine receptor CXCR5 on T cells of myasthenia gravis patients. *J Neuroimmunol* (2005) 170:172–8. doi:10.1016/j.jneuroim.2005.09.001
159. Zhang C-J, Gong Y, Zhu W, Qi Y, Yang C-S, Fu Y, et al. Augmentation of circulating follicular helper T cells and their impact on autoreactive B cells in myasthenia gravis. *J Immunol* (2016) 197:2610–7. doi:10.4049/jimmunol.1500725
160. Luo C, Li Y, Liu W, Feng H, Wang H, Huang X, et al. Expansion of circulating counterparts of follicular helper T cells in patients with myasthenia gravis. *J Neuroimmunol* (2013) 256:55–61. doi:10.1016/j.jneuroim.2012.12.001
161. Yang Y, Zhang M, Ye Y, Ma S, Fan L, Li Z. High frequencies of circulating Tfh-Th17 cells in myasthenia gravis patients. *Neurol Sci* (2017) 38:1599–608. doi:10.1007/s10072-017-3009-3
162. Wen Y, Yang B, Lu J, Zhang J, Yang H, Li J. Imbalance of circulating CD4(+) CXCR5(+)FOXP3(+) Tfr-like cells and CD4(+)CXCR5(+)FOXP3(-) Tfh-like cells in myasthenia gravis. *Neurosci Lett* (2016) 630:176–82. doi:10.1016/j.neulet.2016.07.049
163. Zhang M, Zhou Y, Guo J, Li H, Tian F, Gong L, et al. Thymic TFH cells involved in the pathogenesis of myasthenia gravis with thymoma. *Exp Neurol* (2014) 254:200–5. doi:10.1016/j.expneurol.2014.01.024
164. Song Y, Zhou L, Miao F, Chen G, Zhu Y, Gao X, et al. Increased frequency of thymic T follicular helper cells in myasthenia gravis patients with thymoma. *J Thorac Dis* (2016) 8:314–22. doi:10.21037/jtd.2016.03.03
165. Kamisawa T, Zen Y, Pillai S, Stone JH. IgG4-related disease. *Lancet Lond Engl* (2015) 385:1460–71. doi:10.1016/S0140-6736(14)60720-0
166. Kubo S, Nakayama S, Zhao J, Yoshikawa M, Miyazaki Y, Nawata A, et al. Correlation of T follicular helper cells and plasmablasts with the development of organ involvement in patients with IgG4-related disease. *Rheumatology (Oxford)* (2018) 57:514–24. doi:10.1093/rheumatology/kex455
167. Kamekura R, Takano K, Yamamoto M, Kawata K, Shigehara K, Jitsukawa S, et al. Cutting edge: a critical role of lesional T follicular helper cells in the pathogenesis of IgG4-related disease. *J Immunol* (2017) 199:2624–9. doi:10.4049/jimmunol.1601507
168. Akiyama M, Suzuki K, Yamaoka K, Yasuoka H, Takeshita M, Kaneko Y, et al. Number of circulating follicular helper 2 T cells correlates with IgG4 and interleukin-4 levels and plasmablast numbers in IgG4-related disease. *Arthritis Rheumatol* (2015) 67:2476–81. doi:10.1002/art.39209
169. Akiyama M, Yasuoka H, Yamaoka K, Suzuki K, Kaneko Y, Kondo H, et al. Enhanced IgG4 production by follicular helper 2 T cells and the involvement of follicular helper 1 T cells in the pathogenesis of IgG4-related disease. *Arthritis Res Ther* (2016) 18:167. doi:10.1186/s13075-016-1064-4
170. Grados A, Ebbo M, Piperoglou C, Groh M, Regent A, Samson M, et al. T cell polarization toward TH2/TFH2 and TH17/TFH17 in patients with IgG4-related disease. *Front Immunol* (2017) 8:235. doi:10.3389/fimmu.2017.00235
171. Zaidan M, Cervera-Pierot P, de Seigneux S, Dahan K, Fabiani B, Callard P, et al. Evidence of follicular T-cell implication in a case of IgG4-related systemic disease with interstitial nephritis. *Nephrol Dial Transplant* (2011) 26:2047–50. doi:10.1093/ndt/gfr097
172. Maehara T, Moriyama M, Nakashima H, Miyake K, Hayashida J-N, Tanaka A, et al. Interleukin-21 contributes to germinal centre formation and immunoglobulin G4 production in IgG4-related dacryoadenitis and sialoadenitis, so-called Mikulicz's disease. *Ann Rheum Dis* (2012) 71:2011–9. doi:10.1136/annrheumdis-2012-201477
173. Yang H, Wei R, Liu Q, Shi Y, Li J. Frequency and distribution of CD4+CXCR5+ follicular B helper T cells within involved tissues in IgG4-related ophthalmic disease. *Mol Med Rep* (2017) 16:9512–20. doi:10.3892/mmr.2017.7780
174. Zhao Y, Lutalo PMK, Thomas JE, Sangle S, Choong LM, Tyler JR, et al. Circulating T follicular helper cell and regulatory T cell frequencies

- are influenced by B cell depletion in patients with granulomatosis with polyangiitis. *Rheumatology (Oxford)* (2014) 53:621–30. doi:10.1093/rheumatology/ket406
175. Liu D, Liu J, Wang J, Liu C, Yang S, Jiang Y. Distinct phenotypic subpopulations of circulating CD4+CXCR5+ follicular helper T cells in children with active IgA vasculitis. *BMC Immunol* (2016) 17:40. doi:10.1186/s12865-016-0176-6
 176. Xie J, Liu Y, Wang L, Ruan G, Yuan H, Fang H, et al. Expansion of circulating T follicular helper cells in children with acute Henoch-Schönlein purpura. *J Immunol Res* (2015) 2015:742535. doi:10.1155/2015/742535
 177. Zhang Z, Zhao S, Zhang L, Crew R, Zhang N, Sun X, et al. A higher frequency of CD4(+)CXCR5(+) T follicular helper cells in patients with newly diagnosed Henoch-Schönlein purpura nephritis. *Int Immunopharmacol* (2016) 32:8–15. doi:10.1016/j.intimp.2015.12.037
 178. Liu D, Liu J, Wang J, Guo L, Liu C, Jiang Y, et al. Distribution of circulating T follicular helper cell subsets is altered in immunoglobulin A vasculitis in children. *PLoS One* (2017) 12:e0189133. doi:10.1371/journal.pone.0189133
 179. Xie J, Cui D, Liu Y, Jin J, Tong H, Wang L, et al. Changes in follicular helper T cells in idiopathic thrombocytopenic purpura patients. *Int J Biol Sci* (2015) 11:220–9. doi:10.7150/ijbs.10178
 180. Zhu X, Ma D, Zhang J, Peng J, Qu X, Ji C, et al. Elevated interleukin-21 correlated to Th17 and Th1 cells in patients with immune thrombocytopenia. *J Clin Immunol* (2010) 30:253–9. doi:10.1007/s10875-009-9353-1
 181. Yao X, Li C, Yang J, Wang G, Li C, Xia Y. Differences in frequency and regulation of T follicular helper cells between newly diagnosed and chronic pediatric immune thrombocytopenia. *Blood Cells Mol Dis* (2016) 61:26–36. doi:10.1016/j.bcmd.2016.06.006
 182. Audia S, Rossato M, Santeoets K, Spijkers S, Wichers C, Bekker C, et al. Splenic TFH expansion participates in B-cell differentiation and antiplatelet-antibody production during immune thrombocytopenia. *Blood* (2014) 124:2858–66. doi:10.1182/blood-2014-03-563445
 183. Audia S, Rossato M, Trad M, Samson M, Santeoets K, Gautheron A, et al. B cell depleting therapy regulates splenic and circulating T follicular helper cells in immune thrombocytopenia. *J Autoimmun* (2017) 77:89–95. doi:10.1016/j.jaut.2016.11.002
 184. Wang Y, Wang L, Yang H, Yuan W, Ren J, Bai Y. Activated circulating T follicular helper cells are associated with disease severity in patients with psoriasis. *J Immunol Res* (2016) 2016:7346030. doi:10.1155/2016/7346030
 185. Niu J, Song Z, Yang X, Zhai Z, Zhong H, Hao F. Increased circulating follicular helper T cells and activated B cells correlate with disease severity in patients with psoriasis. *J Eur Acad Dermatol Venereol* (2015) 29:1791–6. doi:10.1111/jdv.13027
 186. Wang Y, Wang L, Shi Y, Wang F, Yang H, Han S, et al. Altered circulating T follicular helper cell subsets in patients with psoriasis vulgaris. *Immunol Lett* (2017) 181:101–8. doi:10.1016/j.imlet.2016.09.008
 187. Caruso R, Botti E, Sarra M, Esposito M, Stolfi C, Diluvio L, et al. Involvement of interleukin-21 in the epidermal hyperplasia of psoriasis. *Nat Med* (2009) 15:1013–5. doi:10.1038/nm.1995
 188. Szabó K, Gáspár K, Dajnoki Z, Papp G, Fábos B, Szegedi A, et al. Expansion of circulating follicular T helper cells associates with disease severity in childhood atopic dermatitis. *Immunol Lett* (2017) 189:101–8. doi:10.1016/j.imlet.2017.04.010
 189. Hennerici T, Pollmann R, Schmidt T, Seipelt M, Tackenberg B, Möbs C, et al. Increased frequency of T follicular helper cells and elevated interleukin-27 plasma levels in patients with Pemphigus. *PLoS One* (2016) 11:e0148919. doi:10.1371/journal.pone.0148919
 190. Li Q, Liu Z, Dang E, Jin L, He Z, Yang L, et al. Follicular helper T Cells (Tfh) and IL-21 involvement in the pathogenesis of bullous pemphigoid. *PLoS One* (2013) 8:e68145. doi:10.1371/journal.pone.0068145
 191. Kenefick R, Wang CJ, Kapadi T, Wardzinski L, Attridge K, Clough LE, et al. Follicular helper T cell signature in type 1 diabetes. *J Clin Invest* (2015) 125:292–303. doi:10.1172/JCI76238
 192. Xu X, Shi Y, Cai Y, Zhang Q, Yang F, Chen H, et al. Inhibition of increased circulating Tfh cell by anti-CD20 monoclonal antibody in patients with type 1 diabetes. *PLoS One* (2013) 8:e79858. doi:10.1371/journal.pone.0079858
 193. Ferreira RC, Simons HZ, Thompson WS, Cutler AJ, Dopico XC, Smyth DJ, et al. IL-21 production by CD4+ effector T cells and frequency of circulating follicular helper T cells are increased in type 1 diabetes patients. *Diabetologia* (2015) 58:781–90. doi:10.1007/s00125-015-3509-8
 194. Viisanen T, Ihtola E-L, Nääntö-Salonen K, Hyöty H, Nurminen N, Selvenius J, et al. Circulating CXCR5+PD-1+ICOS+ follicular T helper cells are increased close to the diagnosis of type 1 diabetes in children with multiple autoantibodies. *Diabetes* (2017) 66:437–47. doi:10.2337/db16-0714
 195. Zhang N, Tai J, Qu Z, Zhang Z, Zhao S, He J, et al. Increased CD4+CXCR5+T follicular helper cells in diabetic nephropathy. *Autoimmunity* (2016) 49:405–13. doi:10.1080/08916934.2016.1196677
 196. Zhu C, Ma J, Liu Y, Tong J, Tian J, Chen J, et al. Increased frequency of follicular helper T cells in patients with autoimmune thyroid disease. *J Clin Endocrinol Metab* (2012) 97:943–50. doi:10.1210/jc.2011-2003
 197. Zhang J, Ren M, Zeng H, Guo Y, Zhuang Z, Feng Z, et al. Elevated follicular helper T cells and expression of IL-21 in thyroid tissues are involved in the pathogenesis of Graves' disease. *Immunol Res* (2015) 62:163–74. doi:10.1007/s12026-015-8647-z
 198. Aust G, Sittig D, Becherer L, Anderegg U, Schütz A, Lamesch P, et al. The role of CXCR5 and its ligand CXCL13 in the compartmentalization of lymphocytes in thyroids affected by autoimmune thyroid diseases. *Eur J Endocrinol* (2004) 150:225–34. doi:10.1530/eje.0.1500225
 199. Maruoka R, Aoki N, Kido M, Iwamoto S, Nishiura H, Ikeda A, et al. Splenectomy prolongs the effects of corticosteroids in mouse models of autoimmune hepatitis. *Gastroenterology* (2013) 145:209–20.e9. doi:10.1053/j.gastro.2013.03.011
 200. Aoki N, Kido M, Iwamoto S, Nishiura H, Maruoka R, Tanaka J, et al. Dysregulated generation of follicular helper T cells in the spleen triggers fatal autoimmune hepatitis in mice. *Gastroenterology* (2011) 140:1322–1333.e1–5. doi:10.1053/j.gastro.2011.01.002
 201. Kimura N, Yamagiwa S, Sugano T, Settsu T, Tominaga K, Kamimura H, et al. Possible involvement of chemokine C-C receptor 7- programmed cell death-1+ follicular helper T-cell subset in the pathogenesis of autoimmune hepatitis. *J Gastroenterol Hepatol* (2018) 33:298–306. doi:10.1111/jgh.13844
 202. Ma L, Qin J, Ji H, Zhao P, Jiang Y. Tfh and plasma cells are correlated with hypergammaglobulinaemia in patients with autoimmune hepatitis. *Liver Int* (2014) 34:405–15. doi:10.1111/liv.12245
 203. Abe K, Takahashi A, Imaizumi H, Hayashi M, Okai K, Kanno Y, et al. Interleukin-21 plays a critical role in the pathogenesis and severity of type I autoimmune hepatitis. *Springerplus* (2016) 5:777. doi:10.1186/s40064-016-2512-y
 204. Wang L, Sun Y, Zhang Z, Jia Y, Zou Z, Ding J, et al. CXCR5+ CD4+ T follicular helper cells participate in the pathogenesis of primary biliary cirrhosis. *Hepatology* (2015) 61:627–38. doi:10.1002/hep.27306
 205. Wang L, Sun X, Qiu J, Cai Y, Ma L, Zhao P, et al. Increased numbers of circulating ICOS+ follicular helper T and CD38+ plasma cells in patients with newly diagnosed primary biliary cirrhosis. *Dig Dis Sci* (2015) 60:405–13. doi:10.1007/s10620-014-3372-3
 206. Zheng J, Wang T, Zhang L, Cui L. Dysregulation of circulating Tfr/Tfh ratio in primary biliary cholangitis. *Scand J Immunol* (2017) 86:452–61. doi:10.1111/sji.12616
 207. Zhou Z-Q, Tong D-N, Guan J, Li M-F, Feng Q-M, Zhou M-J, et al. Circulating follicular helper T cells presented distinctively different responses toward bacterial antigens in primary biliary cholangitis. *Int Immunopharmacol* (2017) 51:76–81. doi:10.1016/j.intimp.2017.08.004
 208. Wang X, Zhu Y, Zhang M, Hou J, Wang H, Jiang Y, et al. The shifted balance between circulating follicular regulatory T cells and follicular helper T cells in patients with ulcerative colitis. *Clin Sci (Lond)* (2017) 131:2933–45. doi:10.1042/CS20171258
 209. Wang Z, Wang Z, Diao Y, Qian X, Zhu N, Dong W. Circulating follicular helper T cells in Crohn's disease (CD) and CD-associated colorectal cancer. *Tumour Biol* (2014) 35:9355–9. doi:10.1007/s13277-014-2208-2
 210. Yu J, He S, Liu P, Hu Y, Wang L, Wang X, et al. Interleukin-21 promotes the development of ulcerative colitis and regulates the proliferation and secretion of follicular T helper cells in the colitis microenvironment. *Mol Med Rep* (2015) 11:1049–56. doi:10.3892/mmr.2014.2824
 211. Sarra M, Monteleone I, Stolfi C, Fantini MC, Sileri P, Sica G, et al. Interferon-gamma-expressing cells are a major source of interleukin-21 in inflammatory bowel diseases. *Inflamm Bowel Dis* (2010) 16:1332–9. doi:10.1002/ibd.21238

212. Ameratunga R, Brewerton M, Slade C, Jordan A, Gillis D, Steele R, et al. Comparison of diagnostic criteria for common variable immunodeficiency disorder. *Front Immunol* (2014) 5:415. doi:10.3389/fimmu.2014.00415
213. Haymore BR, Mikita CP, Tsokos GC. Common variable immune deficiency (CVID) presenting as an autoimmune disease: role of memory B cells. *Autoimmun Rev* (2008) 7:309–12. doi:10.1016/j.autrev.2007.11.024
214. Wehr C, Kivioja T, Schmitt C, Ferry B, Witte T, Eren E, et al. The EUROclass trial: defining subgroups in common variable immunodeficiency. *Blood* (2008) 111:77–85. doi:10.1182/blood-2007-06-091744
215. Chapel H, Lucas M, Lee M, Bjorkander J, Webster D, Grimbacher B, et al. Common variable immunodeficiency disorders: division into distinct clinical phenotypes. *Blood* (2008) 112:277–86. doi:10.1182/blood-2007-11-124545
216. Patuzzo G, Barbieri A, Tinazzi E, Veneri D, Argentino G, Moretta F, et al. Autoimmunity and infection in common variable immunodeficiency (CVID). *Autoimmun Rev* (2016) 15:877–82. doi:10.1016/j.autrev.2016.07.011
217. Grimbacher B, Hutloff A, Schlesier M, Glocker E, Warnatz K, Dräger R, et al. Homozygous loss of ICOS is associated with adult-onset common variable immunodeficiency. *Nat Immunol* (2003) 4:261–8. doi:10.1038/ni902
218. Salzer U, Maul-Pavicic A, Cunningham-Rundles C, Urschel S, Belohradsky BH, Litzman J, et al. ICOS deficiency in patients with common variable immunodeficiency. *Clin Immunol* (2004) 113:234–40. doi:10.1016/j.clim.2004.07.002
219. Coraglia A, Galassi N, Fernández Romero DS, Juri MC, Felippo M, Malbrán A, et al. Common variable immunodeficiency and circulating TFH. *J Immunol Res* (2016) 2016:4951587. doi:10.1155/2016/4951587
220. Cunill V, Clemente A, Lanio N, Barceló C, Andreu V, Pons J, et al. Follicular T cells from smB-common variable immunodeficiency patients are Skewed toward a Th1 phenotype. *Front Immunol* (2017) 8:174. doi:10.3389/fimmu.2017.00174
221. Unger S, Seidl M, van Schouwenburg P, Rakhmanov M, Bulashevskaya A, Frede N, et al. The TH1 phenotype of follicular helper T cells indicates an IFN- γ -associated immune dysregulation in patients with CD21low common variable immunodeficiency. *J Allergy Clin Immunol* (2018) 141:730–40. doi:10.1016/j.jaci.2017.04.041
222. Tiller T, Tsuiji M, Yurasov S, Velinzon K, Nussenzweig MC, Wardemann H. Autoreactivity in human IgG+ memory B cells. *Immunity* (2007) 26:205–13. doi:10.1016/j.immuni.2007.01.009
223. Cols M, Rahman A, Maglione PJ, Garcia-Carmona Y, Simchoni N, Ko H-BM, et al. Expansion of inflammatory innate lymphoid cells in patients with common variable immune deficiency. *J Allergy Clin Immunol* (2016) 137:1206–15.e6. doi:10.1016/j.jaci.2015.09.013
224. Friedmann D, Keller B, Harder I, Schupp J, Tanriver Y, Unger S, et al. Preferential reduction of circulating innate lymphoid cells type 2 in patients with common variable immunodeficiency with secondary complications is part of a broader immune dysregulation. *J Clin Immunol* (2017) 37:759–69. doi:10.1007/s10875-017-0444-0
225. Zhang L, Wang Y, Shi X, Zou H, Jiang Y. A higher frequency of CD4⁺CXCR5⁺ T follicular helper cells in patients with newly diagnosed IgA nephropathy. *Immunol Lett* (2014) 158:101–8. doi:10.1016/j.imlet.2013.12.004
226. Espinosa-Ortega F, Gómez-Martin D, Santana-De Anda K, Romo-Tena J, Villaseñor-Ovies P, Alcocer-Varela J. Quantitative T cell subsets profile in peripheral blood from patients with idiopathic inflammatory myopathies: tilting the balance towards proinflammatory and pro-apoptotic subsets. *Clin Exp Immunol* (2015) 179:520–8. doi:10.1111/cei.12475
227. Taylor DK, Mittereder N, Kuta E, Delaney T, Burwell T, Dacosta K, et al. T follicular helper-like cells contribute to skin fibrosis. *Sci Transl Med* (2018) 10(431):eaf5307. doi:10.1126/scitranslmed.aaf5307
228. Kolenbrander A, Grewe B, Nemazee D, Überla K, Temchura V. Generation of T follicular helper cells in vitro: requirement for B-cell receptor cross-linking and cognate B- and T-cell interaction. *Immunology* (2018) 153:214–24. doi:10.1111/imm.12834
229. Barnett LG, Simkins HMA, Barnett BE, Korn LL, Johnson AL, Wherry EJ, et al. B cell antigen presentation in the initiation of follicular helper T cell and germinal center differentiation. *J Immunol* (2014) 192:3607–17. doi:10.4049/jimmunol.1301284
230. Xu H, Li X, Liu D, Li J, Zhang X, Chen X, et al. Follicular T-helper cell recruitment governed by bystander B cells and ICOS-driven motility. *Nature* (2013) 496:523–7. doi:10.1038/nature12058
231. Liu D, Xu H, Shih C, Wan Z, Ma X, Ma W, et al. T-B-cell entanglement and ICOSL-driven feed-forward regulation of germinal centre reaction. *Nature* (2015) 517:214–8. doi:10.1038/nature13803
232. Shulman Z, Gitlin AD, Weinstein JS, Lainez B, Esplugues E, Flavell RA, et al. Dynamic signaling by T follicular helper cells during germinal center B cell selection. *Science* (2014) 345:1058–62. doi:10.1126/science.1257861
233. Fazilleau N, Eisenbraun MD, Malherbe L, Ebright JN, Pogue-Caley RR, McHeyzer-Williams LJ, et al. Lymphoid reservoirs of antigen-specific memory T helper cells. *Nat Immunol* (2007) 8:753–61. doi:10.1038/ni1472
234. Wallin EF, Jolly EC, Suchánek O, Bradley JA, Espéli M, Jayne DRW, et al. Human T-follicular helper and T-follicular regulatory cell maintenance is independent of germinal centers. *Blood* (2014) 124:2666–74. doi:10.1182/blood-2014-07-585976

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2018 Gensous, Charrier, Duluc, Contin-Bordes, Truchetet, Lazaro, Duffau, Blanco and Richez. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



Regulation of T Follicular Helper Cells in Islet Autoimmunity

Isabelle Serr^{1,2} and Carolin Daniel^{1,2*}

¹ Research Group Immune Tolerance in Diabetes, Institute for Diabetes Research, Helmholtz Diabetes Center at Helmholtz Zentrum München, Munich, Germany, ² German Center for Diabetes Research (DZD), Munich, Germany

OPEN ACCESS

Edited by:

Georgia Foustari,
San Raffaele Hospital
(IRCCS), Italy

Reviewed by:

Lucy S. K. Walker,
University College London,
United Kingdom
Joanna Groom,
Walter and Eliza Hall Institute of
Medical Research, Australia

*Correspondence:

Carolin Daniel
carolin.daniel@helmholtz-muenchen.de

Specialty section:

This article was submitted
to T Cell Biology,
a section of the journal
Frontiers in Immunology

Received: 04 May 2018

Accepted: 12 July 2018

Published: 23 July 2018

Citation:

Serr I and Daniel C (2018)
Regulation of T Follicular Helper
Cells in Islet Autoimmunity.
Front. Immunol. 9:1729.
doi: 10.3389/fimmu.2018.01729

T follicular helper (TFH) cells are an integral part of humoral immunity by providing help to B cells to produce high-affinity antibodies. The TFH precursor compartment circulates in the blood and TFH cell dysregulation is implied in various autoimmune diseases including type 1 diabetes (T1D). Symptomatic T1D is preceded by a preclinical phase (indicated by the presence of islet autoantibodies) with a highly variable progression time to the symptomatic disease. This heterogeneity points toward differences in immune activation in children with a fast versus slow progressor phenotype. In the context of T1D, previous studies on TFH cells have mainly focused on the clinically active state of the disease. In this review article, we aim to specifically discuss recent insights on TFH cells in human islet autoimmunity before the onset of symptomatic T1D. Furthermore, we will highlight advances in the field of TFH differentiation and function during human islet autoimmunity. Specifically, we will focus on the regulation of TFH cells by microRNAs (miRNAs), as well as on the potential use of miRNAs as biomarkers to predict disease progression time and as future drug targets to interfere with autoimmune activation.

Keywords: T follicular helper cells, islet autoimmunity, microRNA92a, krueppel-like factor 2, type 1 diabetes

INTRODUCTION

T follicular helper (TFH) cells are a subset of CD4⁺ T cells characterized by the expression of the C-X-C chemokine receptor type 5 (CXCR5) (1–3) and their master transcription factor B-cell lymphoma 6 (BCL6) (4–6) as well as secretion of the cytokine interleukin-21 (IL-21) (7–9). The expression of CXCR5 together with a low expression of C-C chemokine receptor 7 (CCR7) allows these T cells to enter the B cell follicle in the secondary lymphoid organs (10, 11), where they take part in the germinal center reaction. Specifically, TFH cells interact with germinal center B cells to induce maturation, class switching, and the production of high-affinity antibodies and are therefore an integral part of humoral immunity (1–3).

Although their primary point of action is in the lymph nodes, studies have demonstrated that TFH cell precursors can be found in the blood circulation. These circulating TFH precursors are characterized by the expression of CXCR5, high expression of programmed cell death 1 and low expression of CCR7. Furthermore, circulating TFH precursors are clonally related and phenotypically similar to germinal center TFH cells and comprise a memory compartment that can be reactivated and expanded in response to immunization (12). Therefore, changes in the frequency and phenotype of circulating TFH precursors correlate with those of active TFH cells in the lymph nodes during infections (13). Since continuous stimulation of TFH cells with antigen, in the follicles provided by germinal center B cells, is important to maintain high levels of BCL6 (14), circulating TFH precursors display low or intermediate levels of BCL6 (13).

T follicular helper precursor cells can be subdivided into different subsets according to the effector cytokines they express in parallel to IL-21. Three TFH subsets can be distinguished based on their surface expression of CXCR3 and CCR6. Th1-like TFH cells are CXCR3⁺CCR6⁻ and produce IFN γ , Th2-like TFH cells are CXCR3⁻CCR6⁻ and produce IL-4, IL-5, and IL-13, and Th17-like TFH cells are CXCR3⁻CCR6⁺ and secrete IL-17A and IL-22 (15). Whereas Th2- and Th17-like TFH cells can induce naïve B cells to become plasma cells and produce antibodies, Th1-like TFH cells are suggested to lack this ability (15, 16). CXCR3⁺ TFH precursors were shown to correlate with effective vaccination responses by inducing antibody release from pre-existing memory B cells (16). However, also the memory B cell help by CXCR3⁺ TFH precursors is less efficient compared to that of their CXCR3⁻ counterparts (13, 17). Th2- and Th17-like TFH cells do, however, impact differentially on the class switching of B cells, with Th2-like TFH cells promoting rather IgG and IgE responses and Th17-like TFH cells promoting IgG and IgA responses (15). A recent study on prostate cancer suggests that Th2- and Th17-like TFH cells also impact differentially on the subtype of IgG antibodies produced (18).

Because of their integral role in humoral immunity, TFH cells have been studied in depth in the context of vaccination. Their function of inducing high-affinity antibody responses additionally implies a role of TFH cells in the development and progression of autoimmune diseases that are characterized by the presence of autoantibodies.

One such autoimmune disease is type 1 diabetes (T1D). T1D is the most common metabolic disorder in children and its incidence is rising steadily, especially in young children (19). Impairments in immune tolerance mechanisms can lead to the destruction of the pancreatic insulin-producing β -cells and consequently a failure of blood glucose control, making life-long insulin replacement therapy necessary for patients with symptomatic T1D.

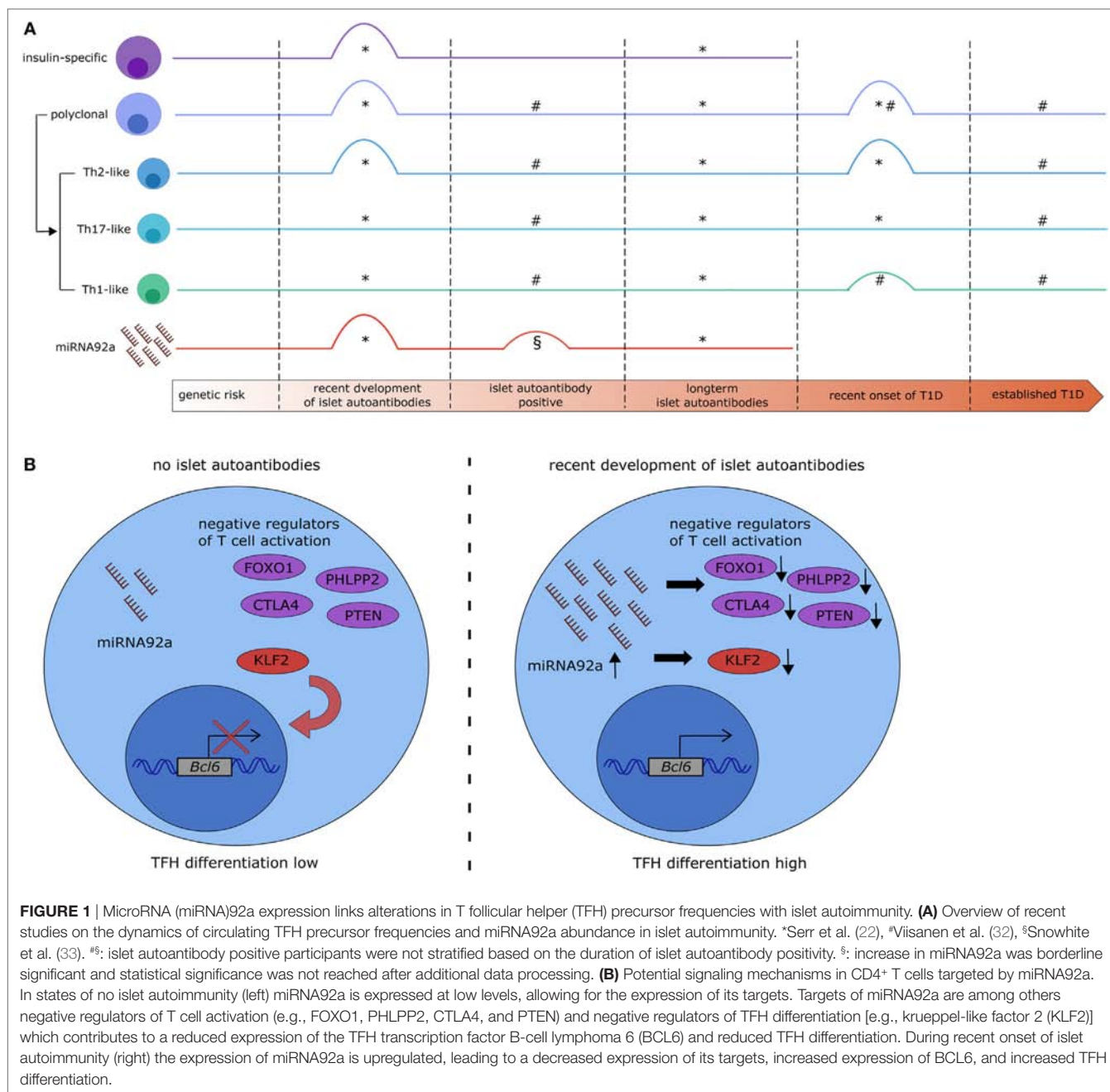
Symptomatic T1D is preceded by a presymptomatic phase (termed islet autoimmunity), characterized by the presence of autoantibodies against islet autoantigens (insulin, insulinoma antigen 2, glutamic acid decarboxylase, zinc transporter 8). The presence of multiple islet autoantibodies increases the life-long risk to develop the symptomatic disease to approximately 100% (20). The time taken for the progression from the development of the first autoantibodies (seroconversion) to the development of the symptomatic disease is, however, very heterogeneous and can range from months (fast progressors) to decades (slow progressors) (20). Accordingly, in our studies, we distinguish different stages of islet autoimmunity: recent onset of islet autoimmunity with islet autoantibodies for less than 5 years and long-term islet autoimmunity with islet autoantibodies for more than 10 years without progression to clinical overt T1D (21–23). However, the immunological mechanisms underlying these differences in disease progression remain poorly understood (24).

TFH CELLS IN PRESYMPTOMATIC T1D

Alterations in the frequency or function of TFH precursor populations in the peripheral blood have been implicated in various autoimmune disorders, including systemic lupus erythematosus

and T1D (25–27). Regarding T1D, Keneflick et al. have demonstrated in a transgenic TCR model that the transfer of TFH cells can induce diabetes. Specifically, they transferred ovalbumin-specific CXCR5⁺ or CXCR5⁻CD4⁺ T cells into recipient mice expressing ovalbumin under the insulin promoter in the β -cells and observed a significant increase in diabetes incidence in mice receiving CXCR5⁺CD4⁺ T cells (28). Furthermore, Ferreira et al. observed increased IL-21 production by CD4⁺ T cells in T1D patients (29). These previous studies on TFH cells in T1D have focused on symptomatic T1D, which excludes conclusions regarding the involvement of TFH cells in the presymptomatic phase or the progression to clinical T1D. The development of multiple islet autoantibodies characterizes the onset of presymptomatic T1D. The important contribution of TFH cells to humoral immunity therefore implicates an involvement of these cells also in disease onset and progression. Accordingly, we found insulin-specific and polyclonal TFH precursor frequencies to be increased during recent onset of islet autoimmunity. This increase was, however, transient and in children with long-term islet autoimmunity without progression to symptomatic T1D, the TFH precursor frequency was similar to that observed in children without islet autoantibodies (22) (**Figure 1A**). This is in accordance with the observation that children with long-term islet autoimmunity tend to lose their first islet autoantibodies, most commonly insulin autoantibodies (30). Data from birth cohort studies highlight that proinsulin-specific CD4⁺ T cells of children who developed islet autoantibodies show a gene expression signature resembling TFH/TH17 cell responses already very early on in infancy, well before the development of islet autoantibodies (31). In a recent Finnish study, no alterations in circulating TFH precursors were observed in normoglycemic children with multiple islet autoantibodies (32) (**Figure 1A**). However, study participants were not discriminated according to the duration of islet autoantibody positivity. These seemingly divergent results highlight the heterogeneity of T1D and underline the necessity to more precisely discriminate the stages of islet autoimmunity and age of study participants.

Regarding the function of circulating TFH precursors, the analysis of Th1-, Th2-, and Th17-like TFH precursors is relevant, because of differences in their ability to provide B cell help and impact on antibody isotype production (15). Data regarding TFH precursor subsets in autoimmune diseases is limited; however, we reported an increase specifically in the Th2-like TFH subset in children with recent onset of islet autoimmunity and in children with newly diagnosed clinical T1D, whereas Th1- and Th17-like TFH cells were unaltered (22). Although Ig subtypes were not analyzed in our study, previous studies highlighted that Ig isotypes of islet autoantibodies and even IgG subtypes induced in the presymptomatic phase of the disease might influence the disease progression (34–36). Similarly, regarding autoimmune diseases other than T1D, a study by Le Coz et al. highlighted an increase of Th2-like TFH cells, accompanied by a decrease in Th1-like TFH cells in patients with systemic lupus erythematosus (37). In this study, Le Coz et al. demonstrate that IgE levels in the serum of lupus patients correlate with disease activity and are associated with high frequencies of Th2-like TFH cells (37).



MECHANISMS OF TFH INDUCTION IN ISLET AUTOIMMUNITY

The TFH differentiation process is highly complex, involving several steps and factors (25–27). In 2013, two research groups demonstrated an important role of the microRNA17~92 (miRNA17~92) cluster, which is essential for normal TFH development and function in mice (38, 39). miRNAs are small, ~22 nucleotide long, non-coding RNAs which can complementarily bind their target mRNAs in the RNA-induced silencing complex and induce their translational silencing or degradation (40–42). miRNAs usually have a multitude of targets and induce rather

modest regulation (43, 44), enabling them to regulate complex cellular states, such as T cell activation (45, 46) and making them suitable targets for immune modulating therapies.

The miRNA17~92 cluster transcribes six mature miRNAs (miRNA17, miRNA18a, miRNA19a, miRNA19b, miRNA20a, and miRNA92a). The relevance of these miRNAs in autoimmune diseases is highlighted by the fact that overexpression of the cluster leads to autoimmunity and autoantibody production in mice (47). Regarding the role of the cluster in murine TFH cell differentiation, miRNA17~92 regulates differentiation and migration of TFH cells together with Bcl6 by repressing TFH subset inappropriate genes like *retinoid-related orphan receptor α*

(*Rora*) and by regulating signaling molecules important for TFH differentiation and function, such as inducible T cell costimulator (Icos) and phosphatidylinositol-3-kinase (PI3K)/protein kinase B signaling (38, 39). Accordingly, two validated targets of miRNA92a are the *phosphatase and tensin homolog* (*Pten*) and *PH domain and leucine rich repeat protein phosphatase 2* (*Phlpp2*), both negative regulators of PI3K signaling (47, 48). In line with its role in TFH cell differentiation and function, additional confirmed targets for miRNA92a are other negative regulators of T cell activation, such as *forkhead box protein O1* (*Foxo1*) and *cytotoxic T-lymphocyte associated protein 4* (*Ctla4*) (47, 48).

In an miRNA profiling approach to investigate miRNAs in T cells that could be involved in human autoimmune activation, we identified miRNA92a to be significantly increased in CD4⁺ T cells from children with ongoing islet autoimmunity compared to healthy controls (22). Confirmation *via* RT-qPCR highlighted an increase in miRNA92a specifically in T cells from children with recent onset of islet autoimmunity and not in children with long-term islet autoimmunity (Figure 1A). Our analysis demonstrated furthermore that this increase in miRNA92a expression correlates with TFH precursor frequencies in the peripheral blood. Accordingly, the lowest expression of miRNA92a was found in T cells from children with long-term islet autoimmunity.

For the investigation of the role of miRNA92a in human TFH differentiation, *in vitro* TFH induction assays, relying on the stimulation of human naïve CD4⁺ T cells with anti-CD3 and anti-CD28 antibodies in the presence of memory B cells, were established. In line with a role of miRNA92a in TFH induction, human TFH induction was decreased in *in vitro* assays, when miRNA92a activity was blocked, whereas an miRNA92a mimic promoted TFH induction (Figure 1B). In assays with an miRNA92a mimic, negative regulators of T cell activation such as *PTEN*, *PHLPP2*, *FOXO1*, and *CTLA4* that are confirmed targets of miRNA92a, were reduced in their expression (22) (Figure 1B). These findings are in line with previous studies, highlighting that TFH cell differentiation is largely dependent on low levels of FOXO1, maintained either by ICOS-PI3K signaling or by degradation *via* the E3 ubiquitin ligase ITCH (49, 50). miRNA92a mediated TFH induction likewise depends on PI3K signaling, since *in vitro* TFH induction with an miRNA92a mimic is blunted in the presence of a PI3K inhibitor, whereas it is increased when PTEN is inhibited (22). PTEN, as a negative regulator of PI3K signaling, is critically involved in the *de novo* induction of regulatory T cells (Tregs). Accordingly, *in vitro* Treg induction from naïve CD4⁺ T cells was found to be impaired in the presence of an miRNA92a mimic. Moreover, insulin-specific Treg frequencies are reduced in children with recent onset of islet autoimmunity, a disease state where miRNA92a abundance was shown to be significantly enhanced in T cells (21, 22) (Figure 2A).

T follicular helper cell function is largely dependent on their ability to enter the B cell follicle in the lymph nodes. Therefore, molecules that regulate lymphocyte trafficking and homing are important mediators of TFH cell function. One example is krueppel-like factor 2 (KLF2). Lee et al. demonstrated that TFH differentiation is dependent on low levels of Klf2, since Klf2 induces the expression of *sphingosine 1 phosphate receptor 1* (*S1pr1*), which

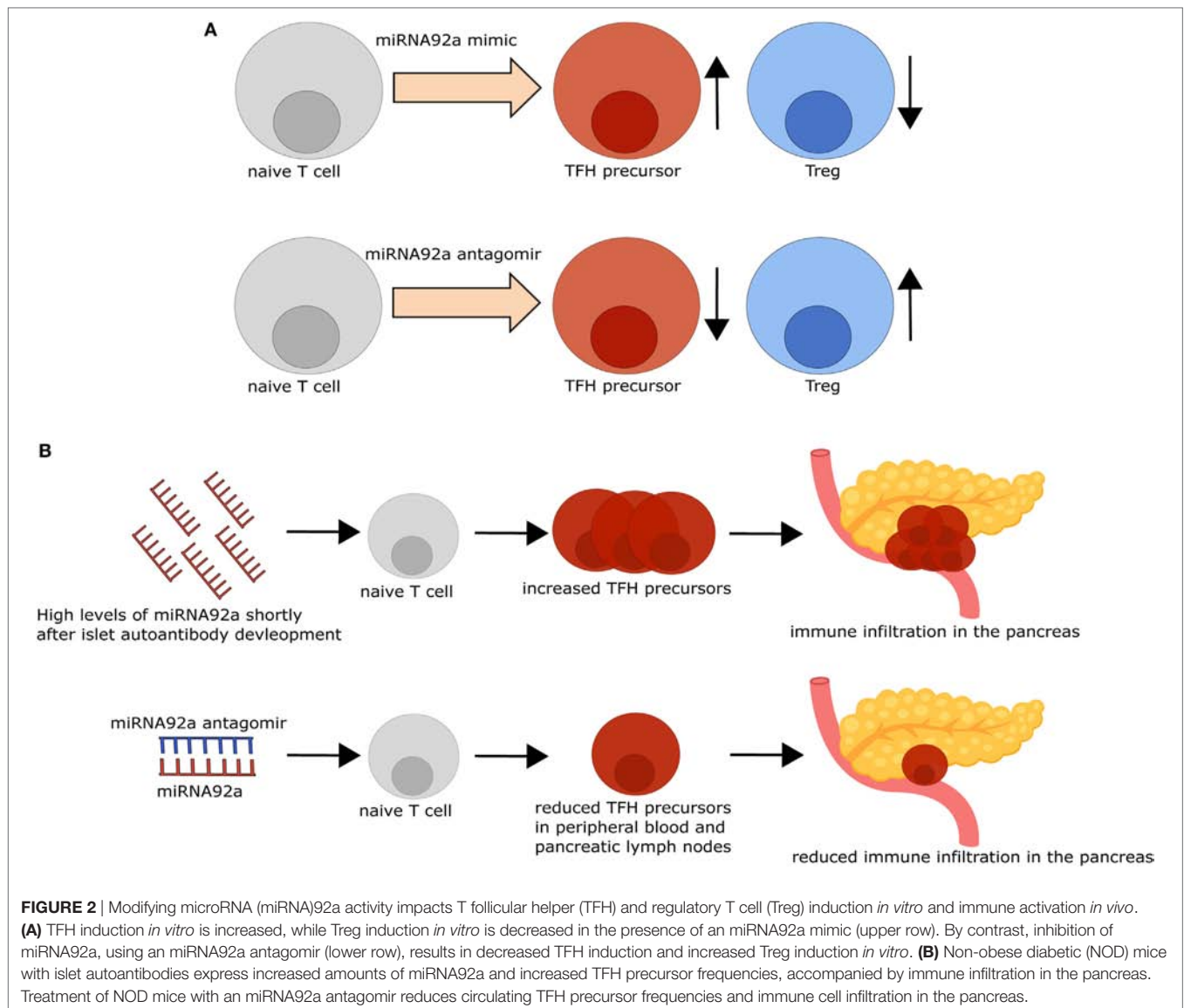
opposes TFH induction (51). Furthermore, Klf2 was shown to inhibit *Bcl6* expression by upregulating B-lymphocyte induced maturation protein 1 (51). Interestingly, our data suggest that *KLF2* can be directly targeted by miRNA92a, since a target site blocker, that inhibits the binding of miRNA92a specifically to *KLF2* abolishes *in vitro* TFH induction (22) (Figure 1B), thereby offering one additional mechanism of miRNA92a-mediated TFH differentiation.

miRNAs AS BIOMARKERS IN ISLET AUTOIMMUNITY

The heterogeneous disease progression from the development of islet autoantibodies to the symptomatic disease necessitates the discovery of biomarkers that will enable a better prediction of the progression time to the clinically active disease. To that end, it remains to be determined, whether changes in miRNA92a expression can also be observed in the serum of children with recent development of islet autoantibodies, or whether the detection of these alterations is limited to the CD4⁺ T cell population. One recent study by Snowwhite et al. aimed at identifying differentially expressed miRNAs in the serum of children with and without autoantibodies. miRNA92a was one of the identified miRNAs that was increased in children with autoantibodies, however, this increase was only borderline significant and not significant after further data processing (33) (Figure 1A). The autoantibody positive children investigated in this study were not stratified based on the duration of autoantibody positivity, which might account for this outcome. The study of longitudinal samples from children at risk of developing T1D will help to assess the usefulness of miRNA92a, TFH cell frequencies, and their respective subsets as biomarkers to predict the progression to clinically overt T1D. More specifically, the analysis of possible correlations between these markers and autoantibody titers or subtypes might be of interest. In this context, a correlation of miRNA92a expression in T cells with TFH precursor frequencies in the blood as well as a modest correlation with insulin autoantibody titers was reported (22). A more detailed analysis of TFH precursor subsets might be especially relevant, because of their divergent functions with respect to providing B cell help and impacting Ig subtype production. Moreover, given the negative impact of high miRNA92a levels on Treg induction, the analysis of Treg frequencies and possible inverse correlations with miRNA92a abundance or TFH subset frequencies can be envisioned. Together, these analyses could be useful to define TFH signature profiles that might serve as biomarkers for assessing T1D disease progression.

TARGETING miRNAs TO INTERFERE WITH AUTOIMMUNE ACTIVATION

miRNAs can function as promising novel potential drug targets, since they can be targeted by small, highly specific oligonucleotides. In this regard, clinical trials for the treatment of hepatitis C virus infections with an miRNA inhibitor have been successfully conducted (52). Targeting specific cell types, especially immune



cells, with miRNA inhibitors is, however, challenging, because of the negative charge of the oligonucleotides which inhibits penetration of the cell membrane (53). Research efforts focus mainly on encapsulation techniques, and various nanoparticles were shown to mediate an efficient uptake of small RNAs by lymphocyte populations (54). Other techniques, targeting T cells more specifically, are, e.g., the use of a single chain CD7 antibody (scFvCD7) fused to an oligonucleotide-nona-arginine peptide (55).

The possibility of altering immune activation and regulation by targeting miRNAs was demonstrated in insulin autoantibody positive non-obese diabetic mice, the most commonly used mouse model for T1D. *I.p.* Application of an miRNA92a antagomir, optimized for *in vivo* use, decreased TFH frequencies and immune activation in the pancreas, accompanied by decreased insulinitis scores and autoantibody titers (22). Furthermore, this decreased immune activation went along with increased frequencies of

Tregs in treated animals, suggesting that, apart from reducing immune activation, inhibition of miRNA92a positively impacts on mediators of T cell tolerance (**Figure 2B**).

The restoration of immune tolerance mechanisms in autoimmune diseases is a long envisioned goal. Since Tregs are important mediators of T cell tolerance in the periphery and can be induced in an antigen-specific fashion, Treg induction could contribute to interfering with the progression of autoimmune activation in autoimmune diseases. This notion is supported by identified associations indicating high frequencies of insulin-specific Tregs accompanied by reduced numbers of insulin-specific TFH precursors in the peripheral blood of children with long-term islet autoimmunity without progression to clinically active T1D. During recent onset of islet autoimmunity, a significant decrease in insulin-specific Treg frequencies was observed accompanied by impaired *in vitro* Treg induction (23). Specifically, during this critical time frame we found an increased sensitivity to antigenic

stimulation in naïve CD4⁺ T cells and reduced expression of negative regulators of T cell activation which can interfere with efficient Treg induction (23). Using miRNAs to tame T cell activation during ongoing islet autoimmunity might therefore open a window of opportunity for improving Treg induction potential in a setting, where the autoimmune process is already in progress. However, the effectiveness of inhibiting miRNA92a to interfere with autoimmune activation and progression to T1D requires long-term *in vivo* studies in animal models of T1D, which are missing so far.

CONCLUSION

Accumulating evidence points toward a role of TFH cells in the development of autoimmune diseases including T1D. During recent onset of islet autoimmunity, children display increased frequencies of TFH precursor cells, specifically Th2-like TFH precursors, whereas this increase is absent in children with long-term islet autoimmunity without overt T1D (22). The analysis of TFH cell frequencies or miRNAs involved in TFH development in longitudinal samples could therefore help to identify biomarkers

in order to improve our ability to predict the progression time to clinically overt T1D, as well as to improve the stratification of respective disease groups. In addition, progress is made regarding the cell type-specific delivery of miRNA inhibitors or mimics. Since miRNAs regulate cellular states, rather than single targets, they compose a new, promising group of future drug targets. In this regard, miRNAs such as miRNA92a that regulate TFH differentiation and function might be targeted to limit immune activation in settings of autoimmunity such as T1D.

AUTHOR CONTRIBUTIONS

IS wrote the manuscript and designed illustrations. CD conceptualized, wrote, and edited the manuscript.

FUNDING

CD is supported by a Research Group at Helmholtz Zentrum München, by the German Center for Diabetes Research (DZD), and through a membership in the CRC1054 of the Deutsche Forschungsgemeinschaft (B11).

REFERENCES

- Breitfeld D, Ohl L, Kremmer E, Ellwart J, Sallusto F, Lipp M, et al. Follicular B helper T cells express CXC chemokine receptor 5, localize to B cell follicles, and support immunoglobulin production. *J Exp Med* (2000) 192(11):1545–52. doi:10.1084/jem.192.11.1545
- Schaerli P, Willmann K, Lang AB, Lipp M, Loetscher P, Moser B. CXC chemokine receptor 5 expression defines follicular homing T cells with B cell helper function. *J Exp Med* (2000) 192(11):1553–62. doi:10.1084/jem.192.11.1553
- Kim CH, Rott LS, Clark-Lewis I, Campbell DJ, Wu L, Butcher EC. Subspecialization of CXCR5⁺ T cells: B helper activity is focused in a germinal center-localized subset of CXCR5⁺ T cells. *J Exp Med* (2001) 193(12):1373–81. doi:10.1084/jem.193.12.1373
- Nurieva RI, Chung Y, Hwang D, Yang XO, Kang HS, Ma L, et al. Generation of T follicular helper cells is mediated by interleukin-21 but independent of T helper 1, 2, or 17 cell lineages. *Immunity* (2008) 29(1):138–49. doi:10.1016/j.immuni.2008.05.009
- Johnston RJ, Poholek AC, DiToro D, Yusuf I, Eto D, Barnett B, et al. Bcl6 and Blimp-1 are reciprocal and antagonistic regulators of T follicular helper cell differentiation. *Science* (2009) 325(5943):1006–10. doi:10.1126/science.1175870
- Yu D, Batten M, Mackay CR, King C. Lineage specification and heterogeneity of T follicular helper cells. *Curr Opin Immunol* (2009) 21(6):619–25. doi:10.1016/j.coi.2009.09.013
- Avery DT, Deenick EK, Ma CS, Suryani S, Simpson N, Chew GY, et al. B cell-intrinsic signaling through IL-21 receptor and STAT3 is required for establishing long-lived antibody responses in humans. *J Exp Med* (2010) 207(1):155–71. doi:10.1084/jem.20091706
- Linterman MA, Beaton L, Yu D, Ramiscal RR, Srivastava M, Hogan JJ, et al. IL-21 acts directly on B cells to regulate Bcl-6 expression and germinal center responses. *J Exp Med* (2010) 207(2):353–63. doi:10.1084/jem.20091738
- Zotos D, Coquet JM, Zhang Y, Light A, D'Costa K, Kallies A, et al. IL-21 regulates germinal center B cell differentiation and proliferation through a B cell-intrinsic mechanism. *J Exp Med* (2010) 207(2):365–78. doi:10.1084/jem.20091777
- Hardtke S, Ohl L, Förster R. Balanced expression of CXCR5 and CCR7 on follicular T helper cells determines their transient positioning to lymph node follicles and is essential for efficient B-cell help. *Blood* (2005) 106(6):1924–31. doi:10.1182/blood-2004-11-4494
- Haynes NM, Allen CDC, Lesley R, Ansel KM, Killeen N, Cyster JG. Role of CXCR5 and CCR7 in follicular Th cell positioning and appearance of a programmed cell death gene-1^{high} germinal center-associated subpopulation. *J Immunol* (2007) 179(8):5099–108. doi:10.4049/jimmunol.179.8.5099
- Heit A, Schmitz F, Gerds S, Flach B, Moore MS, Perkins JA, et al. Vaccination establishes clonal relatives of germinal center T cells in the blood of humans. *J Exp Med* (2017) 214(7):2139–52. doi:10.1084/jem.20161794
- He J, Tsai LM, Leong YA, Hu X, Ma CS, Chevalier N, et al. Circulating precursor CCR7(lo)PD-1(hi) CXCR5(+) CD4(+) T cells indicate Tfh cell activity and promote antibody responses upon antigen reexposure. *Immunity* (2013) 39(4):770–81. doi:10.1016/j.immuni.2013.09.007
- Baumjohann D, Preite S, Reboldi A, Ronchi F, Ansel KM, Lanzavecchia A, et al. Persistent antigen and germinal center B cells sustain T follicular helper cell responses and phenotype. *Immunity* (2013) 38(3):596–605. doi:10.1016/j.immuni.2012.11.020
- Morita R, Schmitt N, Benteibibel SE, Ranganathan R, Bourdery L, Zurawski G, et al. Human blood CXCR5(+)CD4(+) T cells are counterparts of T follicular cells and contain specific subsets that differentially support antibody secretion. *Immunity* (2011) 34(1):108–21. doi:10.1016/j.immuni.2010.12.012
- Benteibibel SE, Lopez S, Obermoser G, Schmitt N, Mueller C, Harrod C, et al. Induction of ICOS+CXCR3+CXCR5⁺ TH cells correlates with antibody responses to influenza vaccination. *Sci Transl Med* (2013) 5(176):176ra132. doi:10.1126/scitranslmed.3005191
- Locci M, Havenar-Daughton C, Landais E, Wu J, Kroenke MA, Arlehamn CL, et al. Human circulating PD-1+CXCR3-CXCR5⁺ memory Tfh cells are highly functional and correlate with broadly neutralizing HIV antibody responses. *Immunity* (2013) 39(4):758–69. doi:10.1016/j.immuni.2013.08.031
- Tan J, Jin X, Zhao R, Wei X, Liu Y, Kong X. Beneficial effect of T follicular helper cells on antibody class switching of B cells in prostate cancer. *Oncol Rep* (2015) 33(3):1512–8. doi:10.3892/or.2014.3684
- Patterson CC, Dahlquist GG, Gyurus E, Green A, Soltesz G, Group ES. Incidence trends for childhood type 1 diabetes in Europe during 1989–2003 and predicted new cases 2005–20: a multicentre prospective registration study. *Lancet* (2009) 373(9680):2027–33. doi:10.1016/S0140-6736(09)60568-7
- Ziegler AG, Rewers M, Simell O, Simell T, Lempainen J, Steck A, et al. Seroconversion to multiple islet autoantibodies and risk of progression to diabetes in children. *JAMA* (2013) 309(23):2473–9. doi:10.1001/jama.2013.6285
- Serr I, Furst RW, Achenbach P, Scherm MG, Gokmen F, Haupt F, et al. Type 1 diabetes vaccine candidates promote human Foxp3(+)Treg induction in humanized mice. *Nat Commun* (2016) 7:10991. doi:10.1038/ncomms10991
- Serr I, Furst RW, Ott VB, Scherm MG, Nikolaev A, Gokmen F, et al. miRNA92a targets KLF2 and the phosphatase PTEN signaling to promote human

- T follicular helper precursors in T1D islet autoimmunity. *Proc Natl Acad Sci U S A* (2016) 113(43):E6659–68. doi:10.1073/pnas.1606646113
23. Serr I, Scherm MG, Zahm AM, Schug J, Flynn VK, Hippich M, et al. A miRNA181a/NFAT5 axis links impaired T cell tolerance induction with autoimmune type 1 diabetes. *Sci Transl Med* (2018) 10(422):eaag1782. doi:10.1126/scitranslmed.aag1782
 24. Achenbach P, Hummel M, Thumer L, Boerschmann H, Hofelmann D, Ziegler AG. Characteristics of rapid vs slow progression to type 1 diabetes in multiple islet autoantibody-positive children. *Diabetologia* (2013) 56(7):1615–22. doi:10.1007/s00125-013-2896-y
 25. Tangye SG, Ma CS, Brink R, Deenick EK. The good, the bad and the ugly – TFH cells in human health and disease. *Nat Rev Immunol* (2013) 13(6):412–26. doi:10.1038/nri3447
 26. Crotty S. T follicular helper cell differentiation, function, and roles in disease. *Immunity* (2014) 41(4):529–42. doi:10.1016/j.immuni.2014.10.004
 27. Scherm MG, Ott VB, Daniel C. Follicular helper T cells in autoimmunity. *Curr Diab Rep* (2016) 16(8):75. doi:10.1007/s11892-016-0770-2
 28. Kenefick R, Wang CJ, Kapadi T, Wardzinski L, Attridge K, Clough LE, et al. Follicular helper T cell signature in type 1 diabetes. *J Clin Invest* (2015) 125(1):292–303. doi:10.1172/JCI76238
 29. Ferreira RC, Simons HZ, Thompson WS, Cutler AJ, Dopico XC, Smyth DJ, et al. IL-21 production by CD4+ effector T cells and frequency of circulating follicular helper T cells are increased in type 1 diabetes patients. *Diabetologia* (2015) 58(4):781–90. doi:10.1007/s00125-015-3509-8
 30. Ziegler AG, Nepom GT. Prediction and pathogenesis in type 1 diabetes. *Immunity* (2010) 32(4):468–78. doi:10.1016/j.immuni.2010.03.018
 31. Heninger A-K, Eugster A, Kuehn D, Buettner F, Kuhn M, Lindner A, et al. A divergent population of autoantigen-responsive CD4+ T cells in infants prior to β cell autoimmunity. *Sci Transl Med* (2017) 9(378):eaaf8848. doi:10.1126/scitranslmed.aaf8848
 32. Viisanen T, Ihanola E-L, Nantö-Salonen K, Hyöty H, Nurminen N, Selvenius J, et al. Circulating CXCR5+PD-1+ICOS+ follicular T helper cells are increased close to the diagnosis of type 1 diabetes in children with multiple autoantibodies. *Diabetes* (2016) 66(2):437–47. doi:10.2337/db16-0714
 33. Snowwhite IV, Allende G, Sosenko J, Pastori RL, Messinger Cayetano S, Pugliese A. Association of serum microRNAs with islet autoimmunity, disease progression and metabolic impairment in relatives at risk of type 1 diabetes. *Diabetologia* (2017) 60(8):1409–22. doi:10.1007/s00125-017-4294-3
 34. Petersen JS, Kulmala P, Clausen JT, Knip M, Dyrberg T; The Childhood Diabetes in Finland Study Group. Progression to type 1 diabetes is associated with a change in the immunoglobulin isotype profile of autoantibodies to glutamic acid decarboxylase (GAD65). *Clin Immunol* (1999) 90(2):276–81. doi:10.1006/clim.1998.4641
 35. Hoppu S, Ronkainen MS, Kimpimäki T, Simell S, Korhonen S, Ilonen J, et al. Insulin autoantibody isotypes during the prediabetic process in young children with increased genetic risk of type 1 diabetes. *Pediatr Res* (2004) 55(2):236–42. doi:10.1203/01.PDR.0000100905.41131.3F
 36. Hoppu S, Härkönen T, Ronkainen MS, Simell S, Hekkälä A, Toivonen A, et al. IA-2 antibody isotypes and epitope specificity during the prediabetic process in children with HLA-conferred susceptibility to type I diabetes. *Clin Exp Immunol* (2006) 144(1):59–66. doi:10.1111/j.1365-2249.2006.03033.x
 37. Le Coz C, Joubin A, Pasquali J-L, Korganow A-S, Dumortier H, Monneaux F. Circulating TFH subset distribution is strongly affected in lupus patients with an active disease. *PLoS One* (2013) 8(9):e75319. doi:10.1371/journal.pone.0075319
 38. Baumjohann D, Kageyama R, Clingan JM, Morar MM, Patel S, de Kouchkovsky D, et al. The microRNA cluster miR-17-92 promotes TFH cell differentiation and represses subset-inappropriate gene expression. *Nat Immunol* (2013) 14(8):840–8. doi:10.1038/ni.2642
 39. Kang SG, Liu WH, Lu P, Jin HY, Lim HW, Shepherd J, et al. MicroRNAs of the miR-17-92 family are critical regulators of T(FH) differentiation. *Nat Immunol* (2013) 14(8):849–57. doi:10.1038/ni.2648
 40. Olsen PH, Ambros V. The lin-4 regulatory RNA controls developmental timing in *Caenorhabditis elegans* by blocking LIN-14 protein synthesis after the initiation of translation. *Dev Biol* (1999) 216(2):671–80. doi:10.1006/dbio.1999.9523
 41. Lewis BP, Shih IH, Jones-Rhoades MW, Bartel DP, Burge CB. Prediction of mammalian MicroRNA targets. *Cell* (2003) 115(7):787–98. doi:10.1016/S0092-8674(03)01018-3
 42. Bagga S, Bracht J, Hunter S, Massirer K, Holtz J, Eachus R, et al. Regulation by let-7 and lin-4 miRNAs results in target mRNA degradation. *Cell* (2005) 122(4):553–63. doi:10.1016/j.cell.2005.07.031
 43. Baek D, Villén J, Shin C, Camargo FD, Gygi SP, Bartel DP. The impact of microRNAs on protein output. *Nature* (2008) 455(7209):64–71. doi:10.1038/nature07242
 44. Selbach M, Schwanhauser B, Thierfelder N, Fang Z, Khanin R, Rajewsky N. Widespread changes in protein synthesis induced by microRNAs. *Nature* (2008) 455(7209):58–63. doi:10.1038/nature07228
 45. Cobb BS, Nesterova TB, Thompson E, Hertweck A, O'Connor E, Godwin J, et al. T cell lineage choice and differentiation in the absence of the RNase III enzyme Dicer. *J Exp Med* (2005) 201(9):1367–73. doi:10.1084/jem.20050572
 46. Cobb BS, Hertweck A, Smith J, O'Connor E, Graf D, Cook T, et al. A role for Dicer in immune regulation. *J Exp Med* (2006) 203(11):2519–27. doi:10.1084/jem.20061692
 47. Xiao C, Srinivasan L, Calado DP, Patterson HC, Zhang B, Wang J, et al. Lymphoproliferative disease and autoimmunity in mice with increased miR-17-92 expression in lymphocytes. *Nat Immunol* (2008) 9(4):405–14. doi:10.1038/ni1575
 48. Jiang P, Rao EY, Meng N, Zhao Y, Wang JJ. MicroRNA-17-92 significantly enhances radioresistance in human mantle cell lymphoma cells. *Radiat Oncol* (2010) 5(1):100. doi:10.1186/1748-717x-5-100
 49. Xiao N, Eto D, Elly C, Peng G, Crotty S, Liu Y-C. The E3 ubiquitin ligase Itch is required for the differentiation of follicular helper T cells. *Nat Immunol* (2014) 15(7):657–66. doi:10.1038/ni.2912
 50. Stone EL, Pepper M, Katayama CD, Kerdiles YM, Lai C-Y, Emslie E, et al. ICOS coreceptor signaling inactivates the transcription factor FOXO1 to promote Tfh cell differentiation. *Immunity* (2015) 42(2):239–51. doi:10.1016/j.immuni.2015.01.017
 51. Lee J-Y, Skon CN, Lee YJ, Oh S, Taylor JJ, Malhotra D, et al. The transcription factor KLF2 restrains CD4+ T follicular helper cell differentiation. *Immunity* (2015) 42(2):252–64. doi:10.1016/j.immuni.2015.01.013
 52. Janssen HLA, Reesink HW, Lawitz EJ, Zeuzem S, Rodriguez-Torres M, Patel K, et al. Treatment of HCV infection by targeting MicroRNA. *N Engl J Med* (2013) 368(18):1685–94. doi:10.1056/NEJMoa1209026
 53. Li Z, Rana TM. Therapeutic targeting of microRNAs: current status and future challenges. *Nat Rev Drug Discov* (2014) 13(8):622–38. doi:10.1038/nrd4359
 54. Peer D, Park EJ, Morishita Y, Carman CV, Shimaoka M. Systemic leukocyte-directed siRNA delivery revealing cyclin D1 as an anti-inflammatory target. *Science* (2008) 319(5863):627–30. doi:10.1126/science.1149859
 55. Kumar P, Ban H-S, Kim S-S, Wu H, Pearson T, Greiner DL, et al. T cell-specific siRNA delivery suppresses HIV-1 infection in humanized mice. *Cell* (2008) 134(4):577–86. doi:10.1016/j.cell.2008.06.034

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2018 Serr and Daniel. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



T Follicular Helper-Like Cells in Inflamed Non-Lymphoid Tissues

Andreas Hutloff*

Chronic Immune Reactions, German Rheumatism Research Centre Berlin (DRFZ), a Leibniz Institute, Berlin, Germany

T and B cell cooperation normally takes place in secondary lymphoid organs (SLO). However, both cell types are also frequently found in inflamed non-lymphoid tissues. Under certain conditions, these infiltrates develop into ectopic lymphoid structures, also known as tertiary lymphoid tissues, which structurally and functionally fully resemble germinal centers (GCs) in SLO. However, tertiary lymphoid tissue is uncommon in most human autoimmune conditions; instead, relatively unstructured T and B cell infiltrates are found. Recent studies have demonstrated that active T and B cell cooperation can also take place in such unstructured aggregates. The infiltrating cells contain a population of T follicular helper (Tfh)-like cells (also designated “peripheral T helper cells”) lacking prototypic Tfh markers like CXCR5 and Bcl-6 but nevertheless expressing high levels of molecules important for B cell help like IL-21 and CD40L. Moreover, Tfh-like cells isolated from inflamed tissues can drive the differentiation of B cells into antibody-secreting cells *in vitro*. These findings are not restricted to experimental animal models but have been reproduced in rheumatoid arthritis and breast cancer patients. At this point, it is unclear whether T and B cell cooperation outside the ordered structure of the GC fully mirrors the reactions in SLO. However, Tfh-like cells in inflamed tissues are certainly important for the local differentiation of B cells into antibody-secreting cells, and should be considered as an important target for the treatment of autoimmune diseases.

Keywords: T follicular helper cells, B cells, inflamed tissue, germinal center, tertiary lymphoid structures

INTRODUCTION

The successful interaction of antigen-specific T and B cells is key to an effective humoral immune response resulting in the generation of high-affinity antibodies and long-term memory B cells. This interaction normally takes place in secondary lymphoid organs (SLO) where T and B cells interact in a temporally and spatially highly organized manner (1). The localization and movement of antigen-specific T and B cells is guided by their expression of chemokine receptors, which respond to defined chemokines locally produced by spatially restricted stromal cells. T follicular helper (Tfh) cells are the CD4⁺ T cell subset providing help for B cells (2). They are characterized by the transcription factor Bcl-6 and high expression of PD-1 and CXCR5, the latter chemokine receptor enabling their movement into the B cell zone. There, in the germinal center (GC) reaction, Tfh cells interact with antigen-specific B cells and drive their affinity maturation and further differentiation by means of the expression of CD40L and IL-21. As affinity maturation by somatic hypermutation always carries the risk of developing autoreactive B cell clones, the GC reaction has several safeguard mechanisms. First, GC B cells by far outnumber Tfh cells. Thereby, Tfh cell help is limited, which fosters selection of B cells with the highest affinity for specific antigen, since only these can efficiently present antigen to Tfh cells (3). This prevents expansion of poly-reactive B cells which also recognize self-antigens with low affinity. Second, the GC itself is further compartmentalized into a light zone, where antigen presentation and selection by Tfh cells occurs, and a dark zone, where B cells proliferate

OPEN ACCESS

Edited by:

Georgia Foustier,
San Raffaele Hospital (IRCCS), Italy

Reviewed by:

Karen Willard-Gallo,
Free University of Brussels, Belgium
Jinfang Zhu,
National Institute of Allergy and
Infectious Diseases (NIAID),
United States

*Correspondence:

Andreas Hutloff,
hutloff@drfz.de

Specialty section:

This article was submitted
to T Cell Biology,
a section of the journal
Frontiers in Immunology

Received: 04 May 2018

Accepted: 11 July 2018

Published: 23 July 2018

Citation:

Hutloff A (2018) T Follicular
Helper-Like Cells in Inflamed
Non-Lymphoid Tissues.
Front. Immunol. 9:1707.
doi: 10.3389/fimmu.2018.01707

and hypermutate their B cell receptor. Cycling of B cells between these two micro-compartments and a rapid degradation of peptide–MHC complexes ensure that B cells are selected according to their actual B cell receptor affinity, and not an “old version” of the BCR retained on the cell surface (4). Finally, high-density presentation of the specific antigen by follicular dendritic cells (FDC) in the light zone provides B cells with an additional signal *via* their antigen receptor. Together, this stringent selection for highest antigen-affinity minimizes the risk of expanding B cells cross-reactive to auto-antigens.

Several recent reports have demonstrated that T and B cell interaction can not only take place outside of SLO but also even in structures completely lacking the highly organized microenvironment of the GC. Inflamed tissues may contain a unique population of non-classical Tfh cells, which can provide help for antigen-specific B cells. Here, I will review these findings and especially discuss their relevance for human autoimmune diseases.

T AND B CELLS IN INFLAMED TISSUES

Under chronic inflammatory conditions like autoimmune or allergic reactions, T and B cells are frequently found as infiltrates in non-lymphoid tissues. There, both cell types substantially contribute to tissue destruction by production of inflammatory cytokines. Since clonally expanded B cell populations in the inflamed tissue outnumber dendritic cells (5) and can efficiently take up low concentrations of antigen due to their high-affinity receptor, they play an important role as antigen-presenting cells and locally promote Th subset differentiation (6, 7). The other half of the interaction, the ability of T cells to provide B cell help, and signals for local B cell differentiation is frequently neglected, although it too contributes to pathology.

ECTOPIC LYMPHOID STRUCTURES (ELS)

Under certain conditions, T and B cell infiltrates in inflamed tissues develop into ELS, also known as tertiary lymphoid tissue (8, 9). These structures anatomically and functionally fully resemble SLO; they are characterized by separated T and B cell zones, the presence of FDC, and high endothelial venules, which enable T and B cells to enter these structures. Within ELS classical GC reactions take place with the presence of CXCR5⁺ Bcl-6⁺ Tfh cells and GC B cells highly proliferating and expressing the cytidine deaminase AID, which is the key enzyme for somatic hypermutation and immunoglobulin class switching. In a mouse model lacking all SLO it was shown that ELS can fully replace their function (10). In human autoimmune diseases, they are considered to play an important role in somatic hypermutation of autoreactive B cells and plasmablast generation directly in the affected tissues (8, 9).

While research on T and B cells in inflamed tissues primarily focused on these ELS, it also became clear that their development requires rather strong stimuli. This was nicely demonstrated in a mouse model where a lung infection with vaccinia virus was directly compared to a bacterial infection with *Pseudomonas aeruginosa* (11). In both cases, prominent lymphoid infiltrates

developed in the lung. However, only in the viral infection model did fully developed, FDC-positive ELS evolve.

Moreover, in human autoimmune diseases, only a fraction of lymphoid infiltrates in inflamed tissues are characterized by fully developed ELS (Table 1). In rheumatoid arthritis patients, where ELS were first described in the synovial membrane of inflamed joints, early studies reported an incidence of fully developed, FDC-containing, and thereby GC-like infiltrates of approximately 25% (12–14). The remaining samples contained either mainly T cells diffusely distributed over the whole tissue or clusters of T and B cells lacking segregation into T and B cell zones and not containing any FDC. However, two more recent studies analyzing larger numbers of samples and more specific parameters came to the conclusion that fully developed, FDC-positive ELS are rather rare in synovial tissue from arthritis patients with a prevalence of only 6–8% (15, 16). This of course might also be related to substantially improved patient treatment regimens in the past years resulting in fewer cases with severely inflamed, end-stage joints. Importantly, patients with fully developed FDC⁺ ELS did not differ from patients with unstructured T and B cell infiltrates regarding several clinical parameters including positivity for rheumatoid factor and anti-citrullinated protein antibodies, suggesting that similar disease processes are occurring in patients with or without FDC⁺ ELS (15, 16).

Also for other autoimmune conditions like systemic lupus erythematosus (SLE), more recent studies revised earlier reports of highly incident fully developed ELS and demonstrated the predominant presence of unstructured, FDC-negative T and B cell aggregates (19). In adult autoimmune myositis, fully developed ELS are completely absent. Nevertheless, B cell receptor sequence analysis from single muscle infiltrates revealed clonally related sequences and locally ongoing somatic hypermutation (24).

Tfh-LIKE CELLS IN INFLAMED TISSUES

In view of the fact that fully developed ELS are rather an exceptional than a common finding in human autoimmune diseases, several recent reports demonstrating that active T and B cell cooperation can also take place in inflamed tissues in unstructured, FDC-negative infiltrates are of high interest. The first indication that highly activated ICOS⁺ CD4⁺ T cells interact with B cells in FDC-negative, unstructured infiltrates and might drive their local differentiation into plasmablasts came from a study

TABLE 1 | Prevalence of fully developed ectopic lymphoid structures (ELS) in human autoimmune diseases.

Disease	Affected organ	Prevalence of ELS ^a (%)	Reference ^b
Rheumatoid arthritis	Synovial joint	6–35	(13–17)
Rheumatoid arthritis	Lung	11	(18)
Systemic lupus erythematosus	Kidney	6	(19)
Sjögren's syndrome	Salivary glands	10–43	(20–23)
Myositis (adult)	Muscle	0	(24)

^aFollicular dendritic cells-positive or with clearly segregated T and B cell zones.

^bConsidering only studies with >15 patients.

analyzing kidney infiltrates in SLE patients (25). In 2008, a Tfh-like but CXCR5- and Bcl-6-negative T cell population was described in the synovial joint of rheumatoid arthritis patients (26). These cells produced the human Tfh signature chemokine CXCL13 and even higher amounts of IL-21 than classical Tfh cells from human tonsil. They were mainly located outside of FDC⁺ GC-like structures and even found in diffuse infiltrates (26, 27). Similar cells could be induced *in vitro* under the influence of TGF- β (28). Only recently, Tfh-like cells from the synovium were characterized in more detail and found to be distinguished by very high expression of PD-1 and chemokine receptors directing migration to inflamed tissues like CCR2 and CCR5 (29). Moreover, it was demonstrated in an *in vitro* T and B cell cooperation assay that these Tfh-like cells can indeed provide B cell help to differentiate B cells into antibody-secreting plasmablasts. Also in the inflamed kidney of lupus nephritis patients, a Tfh-like population expressing high levels of PD-1, ICOS, and CXCR4 was recently described (30). Like in arthritis patients, these cells produced large amounts of IL-21 and were located outside of GC-like structures, but in close contact with B cells.

Similar Tfh-like cells were recently also found in human breast cancer, where their presence was associated with a positive prognosis for the patients (31, 32). These cells lacked the classical Tfh cell-defining markers CXCR5 and Bcl-6 but were positive for other typical markers like PD-1, ICOS, and TIGIT. Furthermore, they were characterized by high expression of IL-21 and CXCL13. Importantly, these cells were not only present in FDC⁺ areas but also in lymphocytic infiltrates near the tumor bed in close contact with tumor-infiltrating B cells.

A more detailed functional analysis of this novel subset of tissue-infiltrating Tfh-like cells became possible by the use of two different lung inflammation mouse models (33, 34). In a house dust mite-induced asthma model, a distinct population of IL-21-producing CD4⁺ T cells was identified in the inflamed lung, where these cells most likely contributed to airway eosinophilia and allergen-specific IgG1 production. These cells expressed high levels of PD-1 but lacked expression of CXCR5 and were also not of Th2 or Th17 lineage origin. In a second lung inflammation mouse model, a similar Tfh-like population was found in lung infiltrates in close contact with GC-like B cells, however, these infiltrates completely lacked any structured GC-like properties (34). High proliferation of lung-resident B cells and the ability of isolated lung T cells to drive differentiation of naive B cells into IgA⁺ plasmablasts *in vitro* demonstrated their B helper potential. These Tfh-like cells were CXCR5- and Bcl-6-negative but expressed even higher levels of PD-1, ICOS, CD40L, and IL-21 than classical Tfh cells in the lung-draining lymph node (34). Apart from the lack of CXCR5 and Bcl-6 expression, their unique characteristics as a cell lineage distinct from classical Tfh cells was demonstrated by the finding that their high expression of PD-1 was independent of ICOS costimulation (5).

Very recently, another Tfh-like cell population was described in a mouse model for systemic sclerosis (35). These PD-1^{high} ICOS^{high} T cells in the skin contributed to fibrosis by their strong production of IL-21. Although these cells were negative for Bcl-6, they expressed significant levels of CXCR5, which clearly discriminates them from the Tfh-like cell populations described above.

GC-LIKE REACTIONS IN INFLAMED TISSUES

These novel findings that a population of Tfh-like cells can provide help to B cells in inflamed tissues in the absence of any ordered GC-like structures of course raise the question, how these T/B interactions can take place, and which consequences arise for the selection of antigen-specific plasma cells and memory B cells.

The presence of FDC has been always considered as a hallmark of the GC. However, as shown in the above-mentioned lung inflammation mouse model, B cells with a phenotype similar to classical GC B cells in SLO (PNA⁺ GL-7⁺ CD38^{low} Bcl-6⁺ AID⁺) can also develop in unstructured, FDC-negative infiltrates in non-lymphoid tissues (34). In this regard, two earlier studies with lymphotoxin β -deficient mice, which lack FDC in all lymphoid organs, are of special interest. Nevertheless, upon immunization these mice developed phenotypically and functionally normal GC (36, 37). Only affinity maturation of the B cell receptor was reported to be delayed in one study (36), whereas the second study demonstrated a shortened persistence of the GC (37). Moreover, in an autoimmune mouse model, B cells undergoing active somatic hypermutation were found outside of FDC-positive B cell follicles (38). Together this shows that other cell types can take over the antigen-presenting function of FDC. In addition to other activated B cells, non-FDC stromal cell populations and macrophages, which are an integral part of leucocytic infiltrates in inflamed tissues, might be good candidates (11, 39).

A second key function of the FDC network in SLO is the spatial organization of the GC. By production of CXCL13, FDC attract both CXCR5⁺ Tfh cells and B cells into the same compartment. Whereas FDC are the main producers of CXCL13 in SLO, other cell types have been shown to take over this function in inflamed tissues (39). At least in the human, the Tfh-like cells in the infiltrates are also a source of CXCL13 (26, 27, 29, 31). However, as Tfh-like cells lack expression of the chemokine receptor CXCR5, recruitment *via* CXCL13 is not an option for the T cells but might be a possibility for B cells, which still express CXCR5 in inflamed tissues, albeit at a reduced level compared to SLO (40). Thereby, other chemokines must be responsible for the striking clusters of T and B cells observed in defined areas of the inflamed tissue. This could be CXCL12, which was shown to be produced by a podoplanin-positive stromal cell population in FDC-negative T and B cell clusters in the inflamed lung (11). Both B cells and Tfh cells express the corresponding chemokine receptor CXCR4. Another good candidate for T and B cell recruitment into inflamed tissues is CXCR3, which is expressed on T and B cells under inflammatory conditions (40, 41). Moreover, very high levels of the corresponding chemokine CXCL10 have been found in the inflamed synovium of rheumatoid arthritis patients (42).

Although recruitment of antigen-specific T and B cells into spatially defined areas of the inflamed tissue can clearly occur without FDC, a highly organized structural segregation into defined T and B cell zones as in SLO is absent (17, 34). On one hand, this facilitates optimal T cell/B cell contact, especially since T cells are not limited as in the classical GC but typically even outnumber B cells. On the other hand, this permanent availability of T cell help, by increasing the chances of T:B interaction, will decrease

the relative competition among B cells for high-affinity antigen. Since the stringent selection for highest antigen affinity like in the classical GC is missing, low-affinity cross- or poly-reactive B cells—including autoreactive B cells—easily become selected.

Another hallmark of GC reactions in SLO is the generation of long-lived plasma cells and memory B cells (1). Whether the plasmablasts numerous found in inflamed tissues later migrate to the bone marrow to become long-lived plasma cells is not known and also difficult to determine experimentally, since the T/B interactions in inflamed tissue are always accompanied by a classical GC reaction in the tissue-draining lymph node. However, it has been clearly demonstrated that antigen-specific Tfh-like cells and B cells generated in the lung tissue could survive locally and in the absence of antigen as long-term tissue-resident memory cells (34). In the same model, memory T and B cells from the immune reaction in the lung were also found as circulating memory cells in spleen and blood. At the moment, it is not clear whether these were cells originally generated in the lung-draining lymph node or whether lung-resident T and B cells have the ability to migrate to other tissues. This is an important question, since it has been discussed for rheumatoid arthritis patients whether autoreactive T and B cells originally generated in the lung might later migrate to joints to induce synovial inflammation (43).

EXTRAFOLLICULAR Tfh CELLS

Another unconventional Tfh cell subset are so called extrafollicular Tfh cells. They were first identified in the spleens of a lupus mouse model as CD4⁺ PSGL-1^{low} CXCR5^{low} CXCR4⁺ T cells producing large amounts of IL-21 and CD40L (44). In contrast to the tissue-resident Tfh-like cells described above, they clearly belong to the Tfh cell lineage as they express Bcl-6 and require it for their development (45). Due to their very low expression of CXCR5, extrafollicular Tfh cells are not located in the GC but at the T/B border where they drive differentiation of extrafollicular

plasmablasts (44). In lupus-prone mice, they are not only found in SLO but also the inflamed kidney where they substantially contribute to pathogenesis (46–48).

CONCLUSION

Recent studies have shown that the ability of B cell help is not exclusive to classical Bcl-6⁺ CXCR5⁺ Tfh cells. Instead, a phenotypically distinct but functionally similar Tfh-like subset can take over this function, especially in inflamed tissues. This is an important notion also in regard to therapeutic strategies to eliminate Tfh cells in chronic inflammatory conditions. Due to their distinct gene expression profile, Tfh-like cells might be refractory to therapies targeting classical Tfh cells like B cell depletion or ICOS costimulation blockade. At the same time, tissue-resident Tfh-like cells are probably the most pathogenic T cell subset, as they select autoreactive B cells in the uncontrolled environment of lymphocytic tissue infiltrates and drive the local differentiation of plasmablasts producing pathogenic antibodies directly in the affected tissues.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and approved it for publication.

ACKNOWLEDGMENTS

Thanks to Laura Bauer and Randall Lindquist for critical reading of the manuscript.

FUNDING

Supported by grants from Deutsche Forschungsgemeinschaft, TRR 130 (P23) and HU 1294/8-1.

REFERENCES

- Mesin L, Ersching J, Vitorica GD. Germinal center B cell dynamics. *Immunity* (2016) 45:471–82. doi:10.1016/j.immuni.2016.09.001
- Vinuesa CG, Linterman MA, Yu D, MacLennan IC. Follicular helper T cells. *Annu Rev Immunol* (2016) 34:335–68. doi:10.1146/annurev-immunol-041015-055605
- Vitorica GD, Schwickert TA, Fooksman DR, Kamphorst AO, Meyer-Hermann M, Dustin ML, et al. Germinal center dynamics revealed by multiphoton microscopy with a photoactivatable fluorescent reporter. *Cell* (2010) 143:592–605. doi:10.1016/j.cell.2010.10.032
- Bannard O, McGowan SJ, Ersching J, Ishido S, Vitorica GD, Shin JS, et al. Ubiquitin-mediated fluctuations in MHC class II facilitate efficient germinal center B cell responses. *J Exp Med* (2016) 213:993–1009. doi:10.1084/jem.20151682
- Vu Van D, Bauer L, Kroczeck RA, Hutloff A. ICOS costimulation differentially affects T cells in secondary lymphoid organs and inflamed tissues. *Am J Respir Cell Mol Biol* (2018). doi:10.1165/rcmb.2017-0309OC
- Leon B, Ballesteros-Tato A, Misra RS, Wojciechowski W, Lund FE. Unraveling effector functions of B cells during infection: the hidden world beyond antibody production. *Infect Disord Drug Targets* (2012) 12:213–21. doi:10.2174/187152612800564437
- Barr TA, Gray M, Gray D. B cells: programmers of CD4 T cell responses. *Infect Disord Drug Targets* (2012) 12:222–31. doi:10.2174/187152612800564446
- Corsiero E, Nerviani A, Bombardieri M, Pitzalis C. Ectopic lymphoid structures: powerhouse of autoimmunity. *Front Immunol* (2016) 7:430. doi:10.3389/fimmu.2016.00430
- Alsughayyir J, Pettigrew GJ, Motalebzadeh R. Spoiling for a fight: B lymphocytes as initiator and effector populations within tertiary lymphoid organs in autoimmunity and transplantation. *Front Immunol* (2017) 8:1639. doi:10.3389/fimmu.2017.01639
- Moyron-Quiroz JE, Rangel-Moreno J, Kusser K, Hartson L, Sprague E, Goodrich S, et al. Role of inducible bronchus associated lymphoid tissue (iBALT) in respiratory immunity. *Nat Med* (2004) 10:927–34. doi:10.1038/nm1091
- Fleige H, Ravens S, Moschovakis GL, Bolter J, Willenzon S, Sutter G, et al. IL-17-induced CXCL12 recruits B cells and induces follicle formation in BALT in the absence of differentiated FDCs. *J Exp Med* (2014) 211:643–51. doi:10.1084/jem.20131737
- Schroder AE, Greiner A, Seyfert C, Berek C. Differentiation of B cells in the nonlymphoid tissue of the synovial membrane of patients with rheumatoid arthritis. *Proc Natl Acad Sci U S A* (1996) 93:221–5. doi:10.1073/pnas.93.1.221
- Klimiuk PA, Goronzy JJ, Bjornsson J, Beckenbaugh RD, Weyand CM. Tissue cytokine patterns distinguish variants of rheumatoid synovitis. *Am J Pathol* (1997) 151:1311–9.
- Takemura S, Braun A, Crowson C, Kurtin PJ, Cofield RH, O'Fallon WM, et al. Lymphoid neogenesis in rheumatoid synovitis. *J Immunol* (2001) 167:1072–80. doi:10.4049/jimmunol.167.2.1072

15. Cantaert T, Kolln J, Timmer T, van der Pouw Kraan TC, Vandooren B, Thurlings RM, et al. B lymphocyte autoimmunity in rheumatoid synovitis is independent of ectopic lymphoid neogenesis. *J Immunol* (2008) 181:785–94. doi:10.4049/jimmunol.181.1.785
16. Thurlings RM, Wijbrandts CA, Mebius RE, Cantaert T, Dinant HJ, van der Pouw-Kraan TC, et al. Synovial lymphoid neogenesis does not define a specific clinical rheumatoid arthritis phenotype. *Arthritis Rheum* (2008) 58:1582–9. doi:10.1002/art.23505
17. Manzo A, Paoletti S, Carulli M, Blades MC, Barone F, Yanni G, et al. Systematic microanatomical analysis of CXCL13 and CCL21 in situ production and progressive lymphoid organization in rheumatoid synovitis. *Eur J Immunol* (2005) 35:1347–59. doi:10.1002/eji.200425830
18. Reynisdottir G, Olsen H, Joshua V, Engstrom M, Forsslund H, Karimi R, et al. Signs of immune activation and local inflammation are present in the bronchial tissue of patients with untreated early rheumatoid arthritis. *Ann Rheum Dis* (2016) 75:1722–7. doi:10.1136/annrheumdis-2015-208216
19. Chang A, Henderson SG, Brandt D, Liu N, Guttikonda R, Hsieh C, et al. In situ B cell-mediated immune responses and tubulointerstitial inflammation in human lupus nephritis. *J Immunol* (2011) 186:1849–60. doi:10.4049/jimmunol.1001983
20. Salomonsson S, Jonsson MV, Skarstein K, Brokstad KA, Hjelmstrom P, Wahren-Herlenius M, et al. Cellular basis of ectopic germinal center formation and autoantibody production in the target organ of patients with Sjogren's syndrome. *Arthritis Rheum* (2003) 48:3187–201. doi:10.1002/art.11311
21. Barone F, Bombardieri M, Manzo A, Blades MC, Morgan PR, Challacombe SJ, et al. Association of CXCL13 and CCL21 expression with the progressive organization of lymphoid-like structures in Sjogren's syndrome. *Arthritis Rheum* (2005) 52:1773–84. doi:10.1002/art.21062
22. Le Pottier L, Devauchelle V, Fautrel A, Daridon C, Saraux A, Youinou P, et al. Ectopic germinal centers are rare in Sjogren's syndrome salivary glands and do not exclude autoreactive B cells. *J Immunol* (2009) 182:3540–7. doi:10.4049/jimmunol.0803588
23. Maehara T, Moriyama M, Hayashida JN, Tanaka A, Shinozaki S, Kubo Y, et al. Selective localization of T helper subsets in labial salivary glands from primary Sjogren's syndrome patients. *Clin Exp Immunol* (2012) 169:89–99. doi:10.1111/j.1365-2249.2012.04606.x
24. Salajegheh M, Pinkus JL, Amato AA, Morehouse C, Jallal B, Yao Y, et al. Permissive environment for B-cell maturation in myositis muscle in the absence of B-cell follicles. *Muscle Nerve* (2010) 42:576–83. doi:10.1002/mus.21739
25. Hutloff A, Buchner K, Reiter K, Baelde HJ, Odendahl M, Jacobi A, et al. Involvement of inducible costimulator in the exaggerated memory B cell and plasma cell generation in systemic lupus erythematosus. *Arthritis Rheum* (2004) 50:3211–20. doi:10.1002/art.20519
26. Manzo A, Vitolo B, Humby F, Caporali R, Jarrossay D, Dell'Accio F, et al. Mature antigen-experienced T helper cells synthesize and secrete the B cell chemoattractant CXCL13 in the inflammatory environment of the rheumatoid joint. *Arthritis Rheum* (2008) 58:3377–87. doi:10.1002/art.23966
27. Kobayashi S, Murata K, Shibuya H, Morita M, Ishikawa M, Furu M, et al. A distinct human CD4+ T cell subset that secretes CXCL13 in rheumatoid synovium. *Arthritis Rheum* (2013) 65:3063–72. doi:10.1002/art.38173
28. Kobayashi S, Watanabe T, Suzuki R, Furu M, Ito H, Ito J, et al. TGF-beta induces the differentiation of human CXCL13-producing CD4(+) T cells. *Eur J Immunol* (2016) 46:360–71. doi:10.1002/eji.201546043
29. Rao DA, Gurish MF, Marshall JL, Slowikowski K, Fonseka CY, Liu Y, et al. Pathologically expanded peripheral T helper cell subset drives B cells in rheumatoid arthritis. *Nature* (2017) 542:110–4. doi:10.1038/nature20810
30. Liarski VM, Kaverina N, Chang A, Brandt D, Yanez D, Talasnik L, et al. Cell distance mapping identifies functional T follicular helper cells in inflamed human renal tissue. *Sci Transl Med* (2014) 6:230ra246. doi:10.1126/scitranslmed.3008146
31. Gu-Trantien C, Migliori E, Buisseret L, de Wind A, Brohee S, Garaud S, et al. CXCL13-producing TFH cells link immune suppression and adaptive memory in human breast cancer. *JCI Insight* (2017) 2:e91487. doi:10.1172/jci.insight.91487
32. Gu-Trantien C, Loi S, Garaud S, Equeter C, Libin M, de Wind A, et al. CD4(+) follicular helper T cell infiltration predicts breast cancer survival. *J Clin Invest* (2013) 123:2873–92. doi:10.1172/JCI67428
33. Coquet JM, Schuijs MJ, Smyth MJ, Deswarte K, Beyaert R, Braun H, et al. Interleukin-21-producing CD4(+) T cells promote type 2 immunity to house dust mites. *Immunity* (2015) 43:318–30. doi:10.1016/j.immuni.2015.07.015
34. Vu Van D, Beier KC, Pietzke LJ, Al Baz MS, Feist RK, Gurka S, et al. Local T/B cooperation in inflamed tissues is supported by T follicular helper-like cells. *Nat Commun* (2016) 7:10875. doi:10.1038/ncomms10875
35. Taylor DK, Mittereder N, Kuta E, Delaney T, Burwell T, Dacosta K, et al. T follicular helper-like cells contribute to skin fibrosis. *Sci Transl Med* (2018) 10:eaf5307. doi:10.1126/scitranslmed.aaf5307
36. Koni PA, Flavell RA. Lymph node germinal centers form in the absence of follicular dendritic cell networks. *J Exp Med* (1999) 189:855–64. doi:10.1084/jem.189.5.855
37. Boulianne B, Le MX, Ward LA, Meng L, Haddad D, Li C, et al. AID-expressing germinal center B cells cluster normally within lymph node follicles in the absence of FDC-M1+ CD35+ follicular dendritic cells but dissipate prematurely. *J Immunol* (2013) 191:4521–30. doi:10.4049/jimmunol.1300769
38. William J, Euler C, Christensen S, Shlomchik MJ. Evolution of autoantibody responses via somatic hypermutation outside of germinal centers. *Science* (2002) 297:2066–70. doi:10.1126/science.1073924
39. Carlsen HS, Baekkevold ES, Morton HC, Haraldsen G, Brandtzaeg P. Monocyte-like and mature macrophages produce CXCL13 (B cell-attracting chemokine 1) in inflammatory lesions with lymphoid neogenesis. *Blood* (2004) 104:3021–7. doi:10.1182/blood-2004-02-0701
40. Nanki T, Takada K, Komano Y, Morio T, Kanegane H, Nakajima A, et al. Chemokine receptor expression and functional effects of chemokines on B cells: implication in the pathogenesis of rheumatoid arthritis. *Arthritis Res Ther* (2009) 11:R149. doi:10.1186/ar2823
41. Qin S, Rottman JB, Myers P, Kassam N, Weinblatt M, Loetscher M, et al. The chemokine receptors CXCR3 and CCR5 mark subsets of T cells associated with certain inflammatory reactions. *J Clin Invest* (1998) 101:746–54. doi:10.1172/JCI1422
42. Patel DD, Zachariah JP, Whichard LP. CXCR3 and CCR5 ligands in rheumatoid arthritis synovium. *Clin Immunol* (2001) 98:39–45. doi:10.1006/clim.2000.4957
43. Chatzidionisyou A, Catrina AI. The lung in rheumatoid arthritis, cause or consequence? *Curr Opin Rheumatol* (2016) 28:76–82. doi:10.1097/BOR.0000000000000238
44. Odegard JM, Marks BR, DiPlacido LD, Poholek AC, Kono DH, Dong C, et al. ICOS-dependent extrafollicular helper T cells elicit IgG production via IL-21 in systemic autoimmunity. *J Exp Med* (2008) 205:2873–86. doi:10.1084/jem.20080840
45. Lee SK, Rigby RJ, Zotos D, Tsai LM, Kawamoto S, Marshall JL, et al. B cell priming for extrafollicular antibody responses requires Bcl-6 expression by T cells. *J Exp Med* (2011) 208:1377–88. doi:10.1084/jem.20102065
46. Odegard JM, DiPlacido LD, Greenwald L, Kashgarian M, Kono DH, Dong C, et al. ICOS controls effector function but not trafficking receptor expression of kidney-infiltrating effector T cells in murine lupus. *J Immunol* (2009) 182:4076–84. doi:10.4049/jimmunol.0800758
47. Teichmann LL, Cullen JL, Kashgarian M, Dong C, Craft J, Shlomchik MJ. Local triggering of the ICOS coreceptor by CD11c(+) myeloid cells drives organ inflammation in lupus. *Immunity* (2015) 42:552–65. doi:10.1016/j.immuni.2015.02.015
48. Bubier JA, Sproule TJ, Foreman O, Spolski R, Shaffer DJ, Morse HC III, et al. A critical role for IL-21 receptor signaling in the pathogenesis of systemic lupus erythematosus in BXSB-Yaa mice. *Proc Natl Acad Sci U S A* (2009) 106:1518–23. doi:10.1073/pnas.0807309106

Conflict of Interest Statement: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2018 Hutloff. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



Follicular Helper T Cells in DiGeorge Syndrome

Adam Klocperk^{1,2*}, Zuzana Paračková¹, Markéta Bloomfield¹, Michal Rataj¹, Jan Pokorný³, Susanne Unger², Klaus Warnatz² and Anna Šedivá¹

¹ Department of Immunology, 2nd Faculty of Medicine, Charles University in Prague and Motol University Hospital, Prague, Czechia, ² Center for Chronic Immunodeficiency (CCI), Medical Center-University of Freiburg, Faculty of Medicine, Freiburg im Breisgau, Germany, ³ Department of Rehabilitation and Sports Medicine, 2nd Faculty of Medicine, Charles University in Prague and Motol University Hospital, Prague, Czechia

OPEN ACCESS

Edited by:

Georgia Foustari,
San Raffaele Hospital
(IRCCS), Italy

Reviewed by:

Andrew R. Gennery,
Newcastle University,
United Kingdom
Davide Montin,
Ospedale Regina
Margherita, Italy

*Correspondence:

Adam Klocperk
adam.klocperk@fnmotol.cz

Specialty section:

This article was submitted
to T Cell Biology,
a section of the journal
Frontiers in Immunology

Received: 05 May 2018

Accepted: 12 July 2018

Published: 23 July 2018

Citation:

Klocperk A, Paračková Z,
Bloomfield M, Rataj M, Pokorný J,
Unger S, Warnatz K and Šedivá A
(2018) Follicular Helper T Cells
in DiGeorge Syndrome.
Front. Immunol. 9:1730.
doi: 10.3389/fimmu.2018.01730

DiGeorge syndrome is an immunodeficiency characterized by thymic dysplasia resulting in T cell lymphopenia. Most patients suffer from increased susceptibility to infections and heightened prevalence of autoimmune disorders, such as autoimmune thrombocytopenia. B cells in DiGeorge syndrome show impaired maturation, with low switched-memory B cells and a wide spectrum of antibody deficiencies or dysgammaglobulinemia, presumably due to impaired germinal center responses. We set out to evaluate circulating follicular helper T cells (cTFHs) in DiGeorge syndrome, as markers of T–B interaction in the germinal centers in a cohort of 17 patients with partial DiGeorge and 21 healthy controls of similar age. cTFHs were characterized as CXCR5⁺CD45RA[−] CD4⁺ T cells using flow cytometry. We verify previous findings that the population of memory CD4⁺ T cells is relatively increased in DiGeorge patients, corresponding to low naïve T cells and impaired T cell production in the thymus. The population of CXCR5⁺ memory CD4⁺ T cells (cTFHs) was significantly expanded in patients with DiGeorge syndrome, but only healthy controls and not DiGeorge syndrome patients showed gradual increase of CXCR5 expression on cTFHs with age. We did not observe correlation between cTFHs and serum IgG levels or population of switched memory B cells. There was no difference in cTFH numbers between DiGeorge patients with/without thrombocytopenia and with/without allergy. Interestingly, we show strong decline of PD1 expression on cTFHs in the first 5 years of life in DiGeorge patients and healthy controls, and gradual increase of PD1 and ICOS expression on CD4⁺ T cells in healthy controls later in life. Thus, here, we show that patients with DiGeorge syndrome have elevated numbers of cTFHs, which, however, do not correlate with autoimmunity, allergy, or production of immunoglobulins. This relative expansion of cTFH cells may be a result of impaired T cell development in patients with thymic dysplasia.

Keywords: immunodeficiency, DiGeorge, T cells, follicular helper T cells, PD1, ICOS, memory, thymus

INTRODUCTION

DiGeorge syndrome is a primary immunodeficiency characterized by thymic dysplasia and T-cell lymphopenia (1). Its most common cause is a 3 Mb deletion on the 22nd chromosome (del22q11.2), which among others encompasses also the *TBX* gene, responsible for the formation of thymic anlage, a basic structural foundation of the thymus, and its further fetal development (2). This

failure to develop a proper niche for the generation of mature thymocytes results in T-cell lymphopenia and increased susceptibility to infection in patients with DiGeorge syndrome. Other clinical symptoms of this syndrome include congenital heart disease, hypoparathyroidism, developmental retardation, and an increased prevalence of autoimmune disease (3–7).

The immune system has been studied thoroughly in DiGeorge syndrome, with a specific focus on T cells and their development. While only 1.5% of patients present with complete DiGeorge syndrome and suffer from life-threatening severe T-cell lymphopenia (8), even patients with partial DiGeorge syndrome show T-cell lymphopenia and decrease of thymic output with low naïve T cells, recent thymic emigrant T cells reflected by low number of T-cell receptor excision circles (4, 9, 10). The impaired T-cell development is further shown to cause oligoclonality within the T-cell compartment (11). Taken together with information on the humoral immune compartment in DiGeorge syndrome patients, including impaired response to vaccination, hypogammaglobulinemia (12, 13), and dysfunctional maturation of B-cells (4, 14, 15), these findings reflect the dysregulation of T–B-cell interactions in DiGeorge syndrome.

The principal subset of T cells crucial for the proper development of germinal center response, B-cell class-switching, and establishment of humoral memory are the follicular helper T cells. These cells are characterized by expression of chemokine receptor CXCR5, which allows their homing along the CXCL13 chemokine gradient produced mainly by follicular dendritic cells in germinal centers (16), thus ensuring their temporospatial colocalization with naïve B cells during the germinal center response to antigen. TFH cells produce IL-21 and express B-cell costimulatory molecules such as ICOSL, CD40L, and others, which promote B-cell proliferation, affinity maturation, and class-switching (17). While TFHs are mostly present in the secondary lymphoid organs, the peripheral blood contains a small population of cells that are generally accepted to be the circulating counterparts of TFH cells [thus circulating follicular helper T cells (cTFHs)] (18, 19). Numerous phenotypic characteristics have been proposed and used, generally including memory marker CD45RO or the absence of CD45RA, the chemokine receptors CXCR5, CCR6, CXCR3, activation/costimulation molecules PD1 and ICOS or the transcription factor Bcl-6. Similarly to changing proportions of naïve vs memory and other T-cell subsets during an individual's life (20), the amount and quality of cTFHs is likely to change over time and has already been shown to decrease in the elderly (21).

There have been several reports describing cTFHs in various primary immunodeficiencies (22–24), but limited information is available on cTFHs in patients with DiGeorge syndrome, even though the combination of dysregulated T-cell development, impaired humoral immunity, and immune dysregulation makes this syndrome a prime candidate for evaluation of cTFH cells. An earlier report by Derfalvi et al. found increased percentage of CXCR5⁺ICOS⁺ CD4 T cells in DiGeorge syndrome patients both below 17 years of age and adults (25). However, no further age-specific resolution was provided or clinical correlation discussed. We, therefore, investigated the cTFH population in pediatric patients with partial DiGeorge syndrome.

MATERIALS AND METHODS

Patients

We present the results of 17 patients with partial DiGeorge syndrome (age 0.5–21 years, mean 7.6 years, 12 females, 5 males), compared to 21 healthy controls (age 0.1–22 years, mean 11.6 years, 9 females, 12 males). Basic patient data are summarized in **Table 1**. All of the patients harbor a del22q11.2 deletion verified through multicolor fluorescent *in situ* hybridization using the DiGeorge/VCFS TUPLE 1/22q Deletion Syndrome LPU004 probe (Cytocell, Cambridge, UK), and at the time of diagnosis, they fulfilled the ESID diagnostic criteria for DiGeorge syndrome. This study was carried out in accordance with the recommendations of the Ethical Committee of the second Faculty of Medicine, Charles University in Prague and University Hospital in Motol, Czech Republic. The protocol was approved by the Ethical Committee. All subjects gave written informed consent in accordance with the Declaration of Helsinki.

Flow Cytometry

Peripheral blood was taken as part of other routine investigations into EDTA-coated tubes, peripheral blood mononuclear cells were isolated using Ficoll-Paque gradient and stained with anti-CD3 Alexa Fluor 700 (clone MEM-57), anti-CD4 Pacific Blue (clone MEM-241, both from Exbio, Czech Republic), anti-CD45RA PE-Cy7 (clone HI100), anti-CXCR5 Alexa Fluor 488 (clone J252D4), anti-PD1 APC (clone EH12.2H7, all from BioLegend, San Diego, CA, USA), and anti-ICOS PE (clone ISA-3, ThermoFisher, MA, USA). Data were acquired on BD FACSaria II cytometer (BD Biosciences, USA) and analyzed using FlowJo VX (FlowJo, LLC, USA) and GraphPad Prism 6 (GraphPad Software, USA). Gating strategy is shown in **Figure 1A**.

RESULTS

Patients with DiGeorge Syndrome Have High Memory CD4⁺ T-Cells due to Comparative Decrease of Naïve CD4⁺ T-Cells

Our cohort of DiGeorge syndrome patients has typically low absolute T cell lymphopenia, which becomes less pronounced with age (**Figure 1A**). Reflecting this finding and we also observe low absolute memory CD4⁺ T cells (**Figure 1B**); however, the low thymic output of naïve T cell compartment (**Figures 1C,D**). This increase starts already at birth, remains constant throughout childhood as shown by other groups (4, 9) results in relative increase of the memory adolescence, and is highly significant (linear regression intercept $p = 0.0002$, slope $p = 0.54$) (**Figure 1C**).

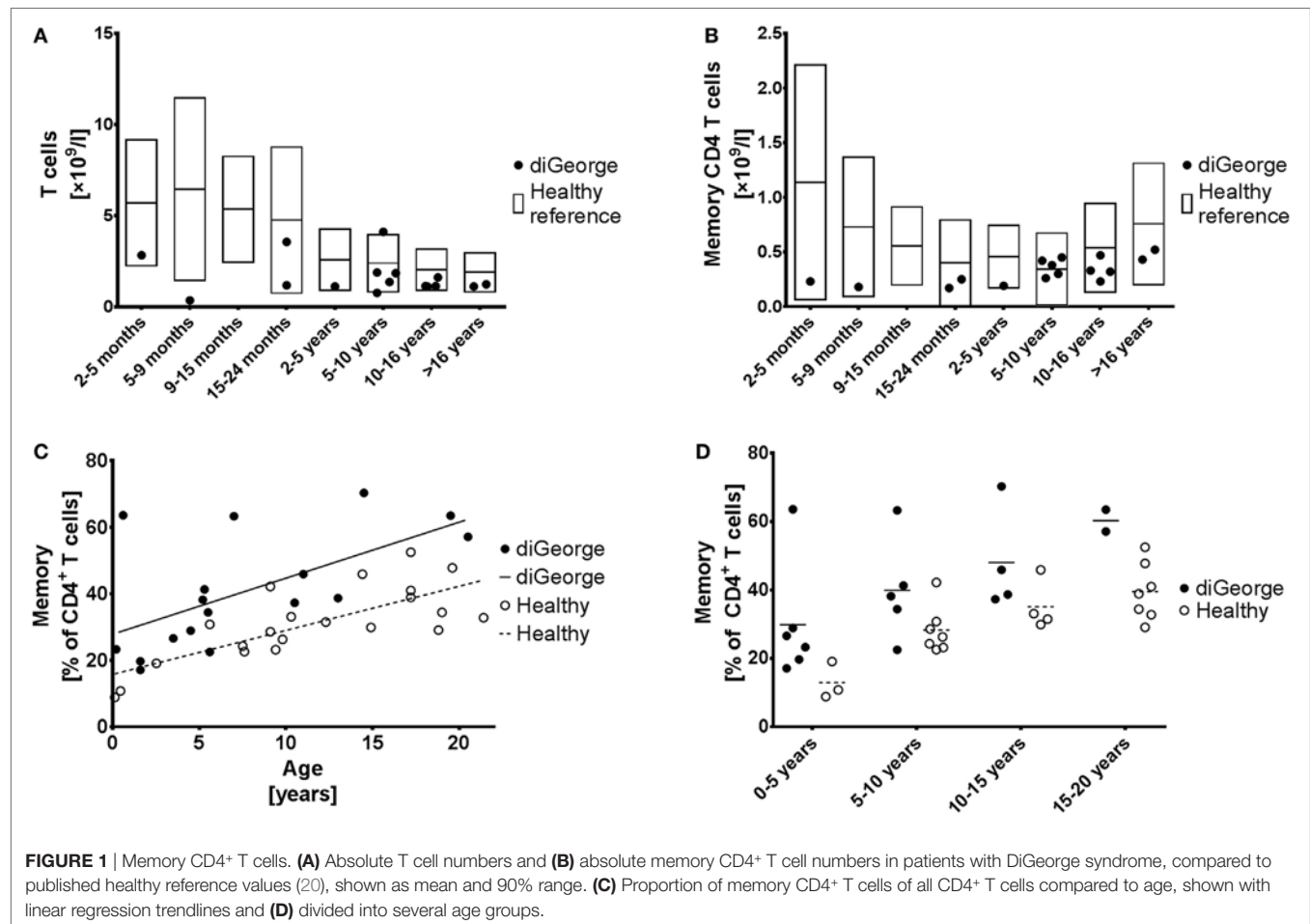
cTFHs Are Expanded in DiGeorge Syndrome

To correct for the relative increase of memory CD4⁺ T cells in DiGeorge, we compared the percentages of CXCR5⁺ memory CD4⁺ T cells (cTFHs) of all memory CD4⁺ T cells (gating strategy shown in **Figure 2A**). We found that patients with DiGeorge

TABLE 1 | Cohort characteristics.

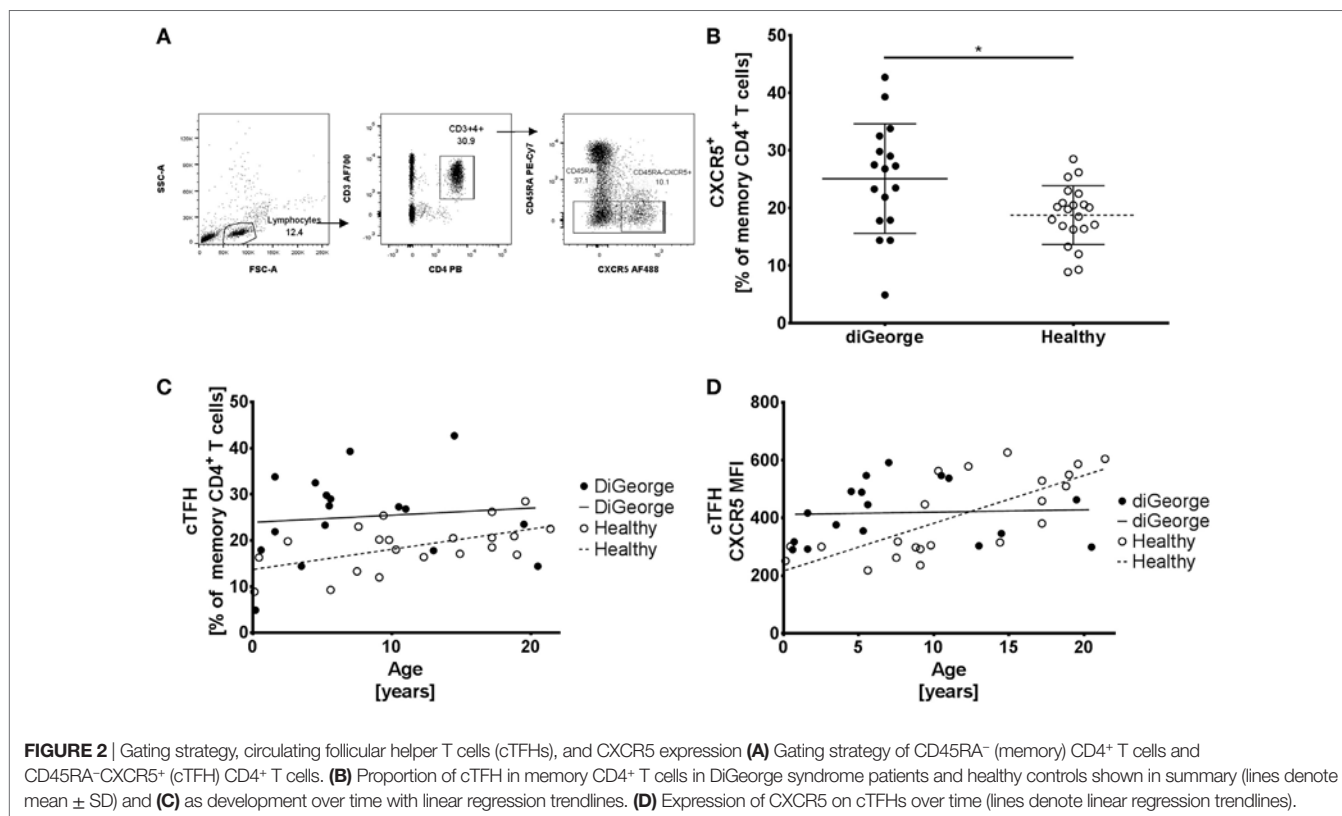
Patient ID	Age (years)	Lymphocytes ($\times 10^9/l$)	CD3 (% of lympho)	CD3 ($\times 10^9/l$)	CD4 (% of lympho)	CD4 ($\times 10^9/l$)	IgG (g/l)	IgM (g/l)	Thrombocytopenia	Allergy	SwM B cells (% of B cells)
Patient 1	0.2	8.32	34	2.83	12	1.00	2.47	0.30	No	No	NA
Patient 2	0.6	1.84	19	0.35	15	0.28	6.64	0.38	No	No	NA
Patient 3	1.6	5.32	67	3.56	28	1.49	9.56	0.59	No	No	NA
Patient 4	1.6	1.91	62	1.18	45	0.86	3.98	0.51	Yes	No	NA
Patient 5	3.5	2.10	53	1.11	34	0.71	12.6	0.28	No	No	8.4
Patient 6	4.5	NA	45	NA	23	NA	11.1	0.78	No	No	NA
Patient 7	5.2	3.49	54	1.88	34	1.19	7.55	0.22	No	No	11.7
Patient 8	5.3	2.67	51	1.36	27	0.72	11.2	0.59	No	No	6.6
Patient 9	5.5	2.55	72	1.84	43	1.10	9.77	0.83	No	Yes	NA
Patient 10	5.6	5.63	73	4.11	33	1.86	11.5	0.62	No	No	10.7
Patient 11	7.0	1.90	40	0.76	22	0.42	9.55	0.65	No	No	5.6
Patient 12	10.5	1.78	62	1.10	34	0.61	12.4	0.88	No	No	14.2
Patient 13	11.0	2.77	58	1.61	26	0.72	11.7	1.11	Yes	No	6.7
Patient 14	13.0	1.89	60	1.13	44	0.83	9.93	0.58	Yes	Yes	9.9
Patient 15	14.5	1.68	67	1.13	40	0.67	17.7	1.62	Yes	Yes	NA
Patient 16	19.5	1.57	71	1.11	43	0.68	10.1	0.41	No	Yes	1.6
Patient 17	20.5	2.01	61	1.23	45	0.90	20.2	3.03	Yes	No	13.3

Table describing patients with DiGeorge syndrome included in this study, including basic laboratory and clinical data.



syndrome have significantly elevated proportion of cTFH within the memory compartment compared to healthy controls (Welch's *t*-test, $p = 0.02$) (**Figure 2B**).

However, whereas this proportion increased with age in healthy controls (linear regression, $p = 0.01$, $R^2 = 0.29$) (**Figure 2C**), there was no significant increase of cTFH/memory CD4⁺ T cell



proportion over time in DiGeorge patients (linear regression, $p = 0.69$, $R^2 = 0.01$) (Figure 2C). This trend is further corroborated by gradual increase of CXCR5 expression on cTFHs in healthy patients (linear regression, $p = 0.0001$, $R^2 = 0.54$) but not DiGeorge patients (linear regression, $p = 0.68$, $R^2 = 0.01$) (Figure 2D).

cTFH Are Not Markers of Humoral Immune Dysregulation in DiGeorge Syndrome

In order to evaluate whether cTFH population reflects the humoral immune dysregulation seen in patients with DiGeorge syndrome, we compared it to serum IgG levels, switched memory B cells, thrombocytopenia, and allergy.

2/17 patients (12%) in our cohort suffered from hypogammaglobulinaemia (Patients 1 and 4), but hypergammaglobulinemia is seen in 7/17 patients (41%) (Patients 5, 6, 8, 10, 12, 15, and 17). However, we observed no correlation between the elevated cTFHs seen in Figure 2B and serum IgG levels (linear regression, $p = 0.19$, $R^2 = 0.11$) (Figure 3A).

Similarly, despite the observed elevated cTFHs, there is a block in B cell maturation with low class-switched memory B cells in DiGeorge syndrome (11, 14). We compared cTFH numbers with switched memory B cells measured as part of previous investigations (0–2 years prior to evaluation of cTFH counts) (14), but saw no correlation (Figure 3B) (linear regression, $p = 0.44$, $R^2 = 0.10$).

To investigate the influence of cTFHs on clinical phenotype of patients, we compared cTFH numbers in patients with/without thrombocytopenia (Figure 3C)—the most commonly seen autoimmune complication in DiGeorge syndrome—and with/without allergy (Figure 3D). There was no difference in cTFHs

between these cohorts, suggesting that cTFHs are not good markers of autoimmunity, allergy, or dysgammaglobulinemia in patients with DiGeorge syndrome.

Expression of PD1 and ICOS Is Preserved on cTFHs of DiGeorge Syndrome Patients

The phenotypic and functional identity of cTFHs has been previously characterized using the extended surface expression of important surface molecules, such as the inhibitory checkpoint-molecule PD1 (26) and the costimulatory receptor ICOS (27, 28). We, therefore, evaluated the surface expression of PD1 and ICOS on cTFHs in DiGeorge patients compared to healthy controls, but saw no significant difference (multiple t -tests with Benjamini FDR approach, PD1 $p = 0.57$, ICOS $p = 0.22$) (Figure 4A).

PD1 and ICOS Expression

Two healthy and three DiGeorge outliers with very high PD1 and ICOS expression on cTFHs can be seen in the summary data (Figure 4A). These are very young (<2 years old) patients and controls. Observing this trend, we evaluated the development of PD1 and ICOS expression in DiGeorge patients and healthy controls with age.

We observed a significant decrease of PD1 expression on cTFHs (linear regression, $p = 0.01$, $R^2 = 0.78$) (Figure 4B), but not CXCR5⁺ memory CD4⁺ T cells (Figure 4C) or CD4⁺ T cells (Figure 4D) in the first 5 years of life in patients with DiGeorge syndrome. This trend seems to be similar in healthy controls, but is not significant, possibly due to low number of samples ($n = 3$) and, therefore, low statistical power.

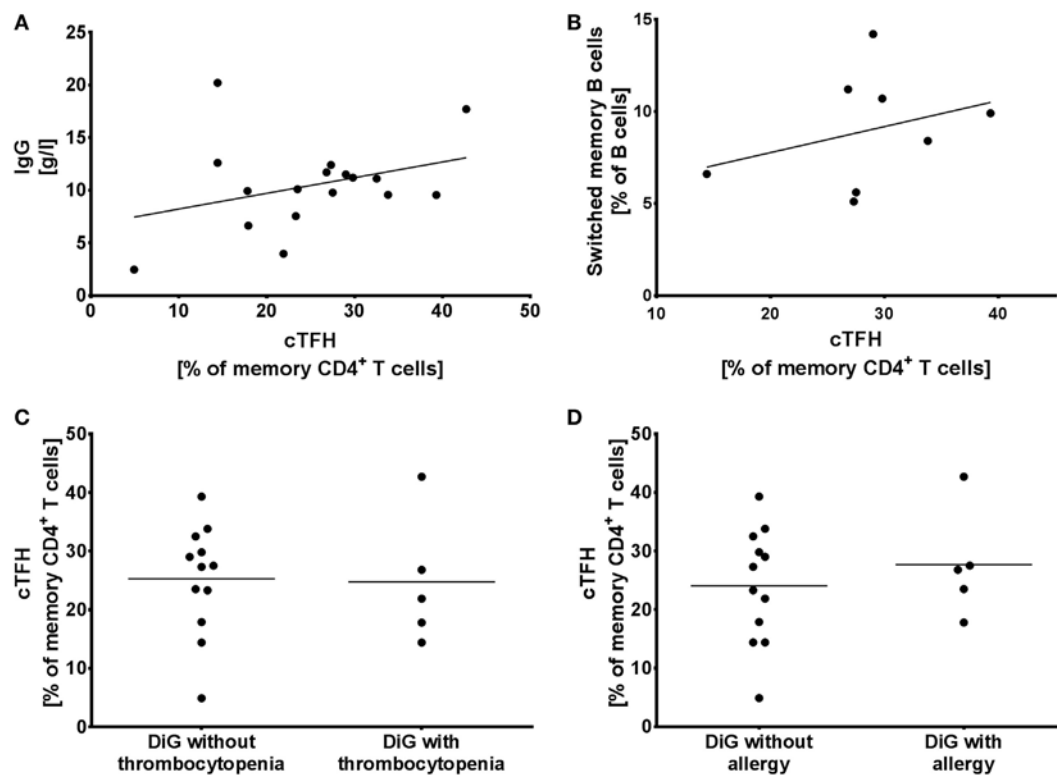


FIGURE 3 | Circulating follicular helper T cells (cTFH) in humoral immune dysregulation, autoimmunity, and allergy. **(A)** Proportion of cTFHs in memory CD4⁺ T cells compared to serum IgG levels and **(B)** switched memory B cells in DiGeorge syndrome patients, shown with linear regression trendlines. **(C)** cTFHs in DiGeorge syndrome patients without/with thrombocytopenia and **(D)** without/with allergy (lines denote mean).

PD1 expression further decreases later in life on cTFHs (linear regression, $p = 0.04$, $R^2 = 0.37$) (Figure 4B), but not CXCR5⁺ memory CD4⁺ T cells (Figure 4C) and CD4⁺ T cells (Figure 4D) in DiGeorge syndrome. In healthy controls, there is no subsequent decrease in cTFHs, but on the contrary, there is significant increase of PD1 expression later in life on both CXCR5⁺ memory CD4⁺ T cells (linear regression, $p = 0.01$, $R^2 = 0.29$) and CD4⁺ T cells (linear regression, $p = 0.0003$, $R^2 = 0.56$), which is not present in DiGeorge patients.

There are no significant changes in ICOS expression on any measured T cell population of DiGeorge syndrome patients (Figures 4F–H). In healthy controls, however, there is a highly significant gradual increase of ICOS expression on CD4⁺ T cells in children older than 5 years. There seems to be a trend similar to the sharp PD1 decrease in first 5 years of life on cTFHs and CXCR5⁺ memory CD4⁺ T cells in healthy controls, but it is not significant.

We verified the observed strong decline of PD1 expression on cTFHs cells during the first 5 years of life on paired samples obtained from two DiGeorge syndrome patients on two consequent visits (Figure 4E).

DISCUSSION

We explore in this manuscript the presence and phenotype of TFH cells in the context of thymic pathology in patients with DiGeorge

syndrome. As our understanding of the immune system grows more detailed, valuable opportunities present themselves at times to elucidate facets of long-known diseases that were not fully understood when those diseases were first described. One such opportunity was the description of the circulating population of follicular helper-like T-cells, which express the chemokine receptor CXCR5 and show functionally and transcriptionally distinct properties.

The inability of immune system in DiGeorge syndrome to produce naïve T-cells in normal quantities has been recognized for a long time and is believed to result from thymic dysplasia. We corroborate these findings on our cohort of pediatric DiGeorge syndrome patients, showing that there is a relative, but not absolute expansion of mature T-cells.

Circulating follicular helper T cells have already been studied in several primary immunodeficiencies, especially with emphasis on T cell B cell cooperation. For example, patients with hyper-IgM syndrome due to CD40L deficiency had low cTFHs (23, 29). The importance of B cells for cTFH generation was also shown in patients with BTK deficiency, who also had low cTFHs (30). Finally, the influence of cTFH on immune system dysregulation was also shown in CVID patients, who suffer from impaired antibody production and low-switched memory B cells, and in whom, elevated Th1 subset of cTFHs was associated with complicated course of the disease (24).

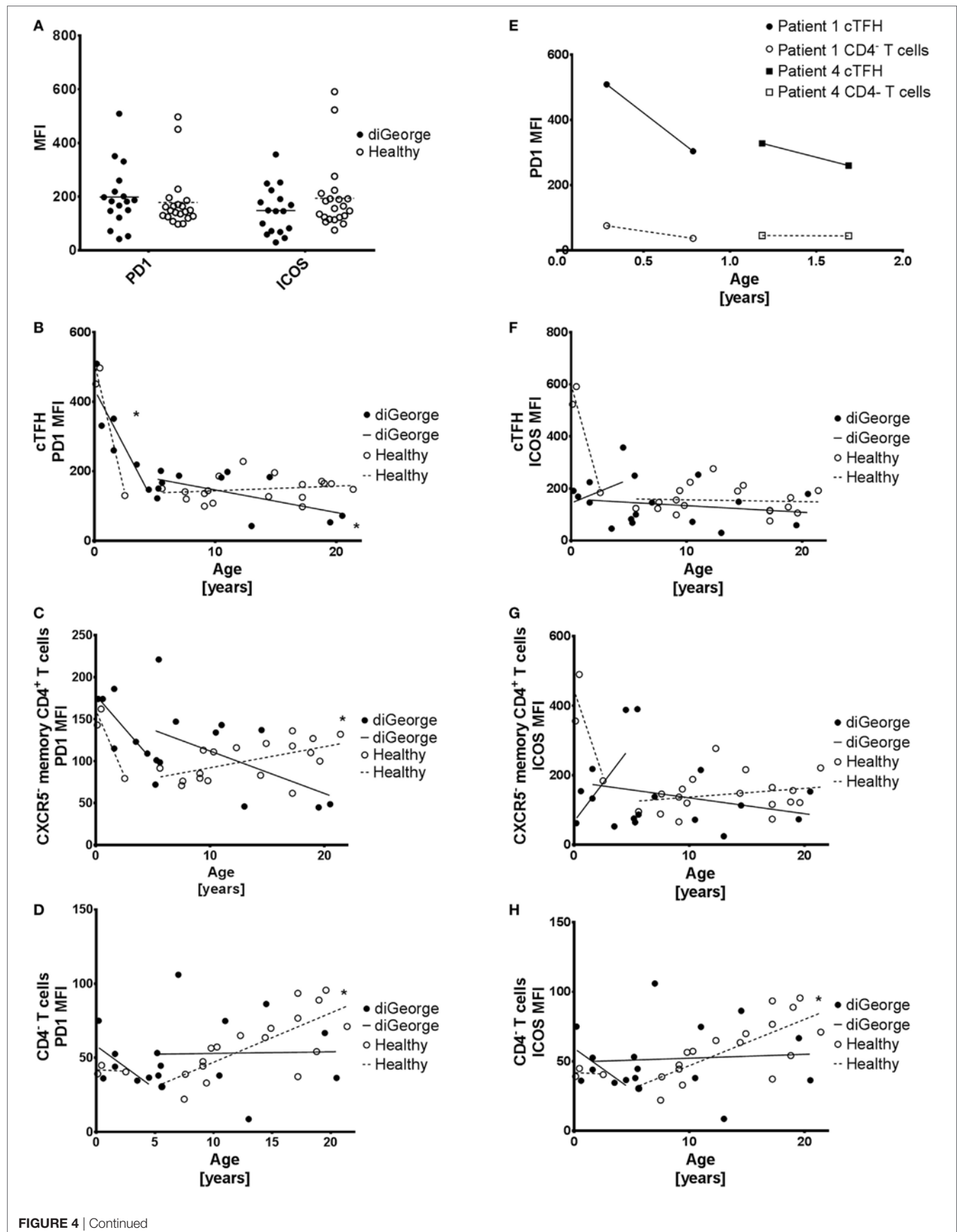


FIGURE 4 | Continued

FIGURE 4 | PD1 and ICOS expression on circulating follicular helper T cells (cTFH), CXCR5⁺ memory CD4⁺ T cells, and CD4⁺ T cells. **(A)** Summary graph showing PD1 and ICOS expression on cTFHs of DiGeorge patients and healthy controls. **(B)** Expression of PD1 on cTFHs, **(C)** CXCR5⁺ memory CD4⁺ T cells, and **(D)** CD4⁺ T cells with linear regression trendlines. * denotes significant correlation. **(E)** Change in PD1 expression over time on paired samples from two patients with DiGeorge syndrome on two consequent visits. **(F)** Expression of ICOS on cTFHs, **(G)** CXCR5⁺ memory CD4⁺ T cells, and **(H)** CD4⁺ T cells with linear regression trendlines. * denotes significant correlation.

However, we show here elevated cTFHs in patients with thymic pathology as part of the DiGeorge syndrome, a finding previously heralded by Derfalvi et al. who also observed elevated bulk and activated cTFHs in DiGeorge syndrome patients (25). Similar trend of elevated cTFH counts was also shown in CVID patients with low-switched memory B-cells (31), and in the context of autoimmune disease such as systemic lupus erythematosus (32), Sjögren's syndrome (33), or rheumatoid arthritis (34). Patients with DiGeorge syndrome also exhibit low class-switched memory B cells as we have shown previously (14); however, in our cohort, we saw no correlation between switched memory B cells and cTFHs, which was also not observed by Derfalvi. While we have not observed any difference in cTFHs between patients with and without history of autoimmune thrombocytopenia, the size of our cohort and lack of patients with other autoimmune complications precludes far-reaching conclusions at this point.

While there have been reports of hypogammaglobulinemia in patients with DiGeorge syndrome (12, 13, 35), which would be expected due to the lack of switched memory B cells, we have shown previously and again in this manuscript that there is a trend toward humoral immune dysregulation and hypergammaglobulinemia in DiGeorge syndrome, especially in adolescents. Although the increased numbers of cTFHs present one possible explanation, we found no correlation between serum IgG levels and cTFH numbers. Such correlation has been shown in the literature for some specific diseases such as IgG4-related disease (36) or rheumatoid arthritis (37), but is otherwise not widely observed, which might reflect the more complex nature of antibody production regulation.

We thus propose that cTFHs are present in patients with DiGeorge syndrome but are dysfunctional in their control and regulation of germinal center response, a hypothesis supported by the observed hypergammaglobulinemia and increased prevalence of autoimmune complications in DiGeorge syndrome and also in our cohort. Thymic dysplasia with loss of central tolerance may lead to production of autoreactive cTFHs resulting in autoimmune complications. Homeostatic proliferation of naïve T cells early in life that has been shown in DiGeorge syndrome (38) may also contribute to the relative expansion of cTFHs, a hypothesis supported by our finding of gradual increase of CXCR5 expression in healthy, but not DiGeorge cTFHs over time, which would indicate early expansion, but impaired long-term maturation of the cTFH compartment.

Finally, we provide data on the changes of PD1 and ICOS expression on various T cell subsets, including cTFH, over time. PD1 is a molecule that has enjoyed a dramatic increase in popularity in recent years, with the advent of checkpoint-blockade treatments in various cancers and with central role in CD8 T-cell exhaustion investigated primarily in chronic viral infections. While much has been documented about the expression of PD1 on cells in adults, there is little to no information available on its expression

pattern during childhood. We observed a strongly increased expression of PD1 on cTFHs, and to lesser extent also CXCR5⁺ memory CD4⁺ T cells, in infants in both DiGeorge syndrome patients and healthy controls. We then show a much slower and gradual increase of PD1 expression in CXCR5⁺ memory CD4⁺ T cells and CD4⁺ T cells in healthy controls, but not DiGeorge syndrome patients, in older childhood and adolescence.

The trend of gradual PD1 expression increase has also been observed in murine CD4⁺ T cells (39) and CD8⁺ T cells (40). High levels of PD1 have also been reported in human neonatal Vdelta2 T cells (41), as well as cord blood Tregs (42). The exact impact of PD1 expression on the function of cTFHs is unclear, however, with both increased (21) and decreased (21) humoral immune response recorded in models with attenuated PD1/PD1L interaction. Interestingly, in their study Derfalvi et al. show increased percentage of CXCR5⁺CCR7^{lo}PD1^{hi} activated cTFH CD4 T cells in both pediatric and adult DiGeorge syndrome patients. Considering the fact that we did not observe increased PD1 expression on DiGeorge cTFHs compared to controls, this finding by Derfalvi et al. may be attributed to higher proportion of bulk cTFHs in CD4 T cells.

In summary, we present here novel information on cTFHs in patients with thymic pathology and primary immunodeficiency underlying DiGeorge syndrome and provide first data on the change in PD1 and ICOS expression on cTFHs and other T cell subsets during childhood. Our work proposes new challenges for investigation in patients with primary immunodeficiency, which could lead to better understanding of the function of cTFHs, as well as temporal development of PD1 and ICOS expression.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the Ethical Committee of the second Faculty of Medicine, Charles University in Prague and University Hospital in Motol, Czech republic. The protocol was approved by the Ethical Committee. All subjects gave written informed consent in accordance with the Declaration of Helsinki.

AUTHOR CONTRIBUTIONS

AK designed the study, performed experiments, analyzed results, and wrote the manuscript. ZP designed the study and performed the experiments. MB and JP assisted with sample acquisition and preparation and provided clinical information. MR performed experiments. SU analyzed the data, interpreted results, and contributed to the discussion. KW contributed to the discussion of data and writing of the manuscript. AŠ interpreted results, assisted with sample acquisition and preparation, and provided clinical information and co-wrote the manuscript. All

authors contributed to manuscript revision, read and approved the submitted version.

FUNDING

The work was financially supported by grants AZV NV18-05-00162 issued by the Czech health research council and Ministry

of Health, Czech republic, GAUK 127315 issued by the Charles University in Prague, Czech republic, as well as institutional support of research organization #00064203 from University Hospital in Motol, Czech republic. The article processing charge was funded by the German Research Foundation (DFG) and the University of Freiburg in the funding programme Open Access Publishing.

REFERENCES

- DiGeorge AM, Lischner HW, Dacou C, Arey JB. Absence of thymus. *Lancet* (1967) 1(7504):1387.
- Driscoll DA, Budarf ML, Emanuel BS. A genetic etiology for DiGeorge syndrome: consistent deletions and microdeletions of 22q11. *Am J Hum Genet* (1992) 50:924–33.
- Gennery AR. Immunological aspects of 22q11.2 deletion syndrome. *Cell Mol Life Sci* (2012) 69:12–27. doi:10.1007/s00018-011-0842-z
- McLean-Tooke A, Barge D, Spickett GP, Gennery AR. Immunologic defects in 22q11.2 deletion syndrome. *J Allergy Clin Immunol* (2008) 122:362–7. doi:10.1016/j.jaci.2008.03.033
- Davies EG. Immunodeficiency in DiGeorge syndrome and options for treating cases with complete athymia. *Front Immunol* (2013) 4:322. doi:10.3389/fimmu.2013.00322
- Šedivá A, Bartůňková J, Zachová R, Hrušák O, Kočárek E, Novotná D, et al. Vývoj imunity u syndromu diGeorge. *Alergie* (2003):1(8).
- Jawad AF, McDonald-McGinn DM, Zackai E, Sullivan KE. Immunologic features of chromosome 22q11.2 deletion syndrome (DiGeorge syndrome/velocardiofacial syndrome). *J Pediatr* (2001) 139:715–23. doi:10.1067/mpd.2001.118534
- Ryan AK, Goodship JA, Wilson DI, Philip N, Levy A, Seidel H, et al. Spectrum of clinical features associated with interstitial chromosome 22q11 deletions: a European collaborative study. *J Med Genet* (1997) 34:798–804. doi:10.1136/jmg.34.10.798
- Lima K, Abrahamson TG, Foelling I, Natvig S, Ryder LP, Olausson RW. Low thymic output in the 22q11.2 deletion syndrome measured by CCR9+CD45RA+ T cell counts and T cell receptor rearrangement excision circles. *Clin Exp Immunol* (2010) 161:98–107. doi:10.1111/j.1365-2249.2010.04152.x
- Frůňková E, Klocperk A, Svaton M, Nováková M, Kotrova M, Kayserova J, et al. The TREC/KREC assay for the diagnosis and monitoring of patients with DiGeorge syndrome. *PLoS One* (2014) 9:e114514. doi:10.1371/journal.pone.0114514
- Zemble R, Prak EL, McDonald K, McDonald-mcgin D, Zackai E, Sullivan K. Secondary immunologic consequences in chromosome 22q11.2 deletion syndrome (DiGeorge syndrome/velocardiofacial syndrome). *Clin Immunol* (2010) 136:409–18. doi:10.1016/j.clim.2010.04.011
- Patel K, Akhter J, Kobrynski L, Benjamin Gathmann MA, Gathman B, Davis O, et al. Immunoglobulin deficiencies: the B-lymphocyte side of DiGeorge Syndrome. *J Pediatr* (2012) 161:950–3. doi:10.1016/j.jpeds.2012.06.018
- Gennery AR, Barge D, O'Sullivan JJ, Flood TJ, Abinun M, Cant AJ. Antibody deficiency and autoimmunity in 22q11.2 deletion syndrome. *Arch Dis Child* (2002) 86:422–5. doi:10.1136/adc.86.6.422
- Klocperk A, Mejstříková E, Kayserová J, Kalina T, Šedivá A. Low marginal zone-like B lymphocytes and natural antibodies characterize skewed B-lymphocyte subpopulations in del22q11 DiGeorge patients. *Clin Immunol* (2015) 161:144–9. doi:10.1016/j.clim.2015.08.013
- Finocchi A, Di Cesare S, Romiti ML, Capponi C, Rossi P, Carsetti R, et al. Humoral immune responses and CD27+ B cells in children with DiGeorge syndrome (22q11.2 deletion syndrome). *Pediatr Allergy Immunol* (2006) 17:382–8. doi:10.1111/j.1399-3038.2006.00409.x
- Ma CS, Deenick EK, Batten M, Tangye SG. The origins, function, and regulation of T follicular helper cells. *J Exp Med* (2012) 209:1241–53. doi:10.1084/jem.20120994
- Breitfeld D, Ohl L, Kremmer E, Ellwart J, Sallusto F, Lipp M, et al. Follicular B helper T cells express CXC chemokine receptor 5, localize to B cell follicles, and support immunoglobulin production. *J Exp Med* (2000) 192:1545–52. doi:10.1084/jem.192.11.1545
- Tangye SG, Ma CS, Brink R, Deenick EK. The good, the bad and the ugly – TFH cells in human health and disease. *Nat Rev Immunol* (2013) 13:412–26. doi:10.1038/nri3447
- Morita R, Schmitt N, Bentebibel SE, Ranganathan R, Bourdery L, Zurawski G, et al. Human blood CXCR5+CD4+ T cells are counterparts of T follicular cells and contain specific subsets that differentially support antibody secretion. *Immunity* (2011) 34:108–21. doi:10.1016/j.immuni.2011.01.009
- Schatorjé EJH, Gemen EFA, Driessen GJA, Leuvenink J, van Hout RWNM, de Vries E. Paediatric reference values for the peripheral T cell compartment. *Scand J Immunol* (2012) 75:436–44. doi:10.1111/j.1365-3083.2012.02671.x
- Herati RS, Reuter MA, Dolfi DV, Mansfield KD, Aung H, Badwan OZ, et al. Circulating CXCR5+ PD-1+ response predicts influenza vaccine antibody responses in young adults but not elderly adults. *J Immunol* (2014) 193:3528–37. doi:10.4049/jimmunol.1302503
- Ma CS. Human T follicular helper cells in primary immunodeficiency: quality just as important as quantity. *J Clin Immunol* (2016) 36:40–7. doi:10.1007/s10875-016-0257-6
- Ma CS, Wong N, Rao G, Avery DT, Torpy J, Hambridge T, et al. Monogenic mutations differentially affect the quantity and quality of T follicular helper cells in patients with human primary immunodeficiencies. *J Allergy Clin Immunol* (2015) 136(4):993–1006.e1. doi:10.1016/j.jaci.2015.05.036
- Unger S, Seidl M, van Schouwenburg P, Rakhmanov M, Bulashevskaya A, Frede N, et al. The TH1 phenotype of follicular helper T cells indicates an IFN- γ -associated immune dysregulation in patients with CD21 low common variable immunodeficiency. *J Allergy Clin Immunol* (2018) 141(2):730–40. doi:10.1016/j.jaci.2017.04.041
- Derfalvi B, Maurer K, McDonald McGinn DM, Zackai E, Meng W, Luning Prak ET, et al. B cell development in chromosome 22q11.2 deletion syndrome. *Clin Immunol* (2016) 163:1–9. doi:10.1016/j.clim.2015.12.004
- Dorfman DM, Brown JA, Shahsafaei A, Freeman GJ. Programmed death-1 (PD-1) is a marker of germinal center-associated T cells and angioimmunoblastic T-cell lymphoma. *Am J Surg Pathol* (2006) 30:802–10. doi:10.1097/01.pas.0000209855.28282.ce
- Choi Y, Kageyama R, Eto D. Bcl6 dependent T follicular helper cell differentiation diverges from effector cell differentiation during priming and depends on the gene Icos. *Immunity* (2011) 34:932–46. doi:10.1016/j.immuni.2011.03.023
- Bauquet AT, Jin H, Paterson AM, Mitsdoerffer M, Ho I, Sharpe AH, et al. Costimulatory molecule ICOS plays a critical role in the development of TH-17 and follicular T-helper cells by regulating c-Maf expression and IL-21 production. *Nat Immunol* (2009) 10:167–75. doi:10.1038/ni.1690
- Bossaller L, Burger J, Draeger R, Grimbacher B, Knoth R, Plebani A, et al. ICOS deficiency is associated with a severe reduction of CXCR5+CD4 germinal center Th cells. *J Immunol* (2006) 177:4927–32. doi:10.4049/jimmunol.177.7.4927
- Boisson B, Wang YD, Bosompem A, Ma CS, Lim A, Kochetkov T, et al. A recurrent dominant negative E47 mutation causes agammaglobulinemia and BCR- B cells. *J Clin Invest* (2013) 123:4781–5. doi:10.1172/JCI71927
- Cunill V, Clemente A, Lanio N, Barceló C, Andreu V, Pons J, et al. Follicular T cells from smB-common variable immunodeficiency patients are skewed toward a Th1 phenotype. *Front Immunol* (2017) 8:174. doi:10.3389/fimmu.2017.00174
- Choi J-Y, Ho JH, Pasoto SG, Bunin V, Kim ST, Carrasco S, et al. Circulating follicular helper-like t cells in systemic lupus erythematosus: association with disease activity. *Arthritis Rheumatol* (2015) 67(4):988–99. doi:10.1002/art.39020
- Szabo K, Papp G, Barath S, Gyimesi E, Szanto A, Zeher M. Follicular helper T cells may play an important role in the severity of primary Sjögren's syndrome. *Clin Immunol* (2013) 147:95–104. doi:10.1016/j.clim.2013.02.024

34. Ma J, Zhu C, Ma B, Tian J, Baidoo SE, Mao C, et al. Increased frequency of circulating follicular helper T cells in patients with rheumatoid arthritis. *Clin Dev Immunol* (2012) 2012:827480. doi:10.1155/2012/827480
35. Junker AK, Driscoll DA. Humoral immunity in DiGeorge syndrome. *J Pediatr* (1995) 127:231–7. doi:10.1016/S0022-3476(95)70300-4
36. Kubo S, Nakayamada S, Zhao J, Yoshikawa M, Miyazaki Y, Nawata A, et al. Correlation of T follicular helper cells and plasmablasts with the development of organ involvement in patients with IgG4-related disease. *Rheumatology* (2017) 57(3):514–24. doi:10.1093/rheumatology/kex455
37. Rao DA, Gurish MF, Marshall JL, Slowikowski K, Fonseca CY, Liu Y, et al. Pathologically expanded peripheral T helper cell subset drives B cells in rheumatoid arthritis. *Nature* (2017) 542:110–4. doi:10.1038/nature20810
38. Ferrando-mart S, Lorente R, Gurbindo D, De Jose MI, Leal M, Muñoz-Fernández MA. Low thymic output, peripheral homeostasis deregulation, and hastened regulatory T cells differentiation in children with 22q11.2 deletion syndrome. *J Pediatr* (2014) 164:882–9. doi:10.1016/j.jpeds.2013.12.013
39. Shimada Y, Hayashi M, Nagasaka Y, Ohno-Iwashita Y, Inomata M. Age-associated up-regulation of a negative co-stimulatory receptor PD-1 in mouse CD4+T cells. *Exp Gerontol* (2009) 44:517–22. doi:10.1016/j.exger.2009.05.003
40. Lee KA, Shin KS, Kim GY, Song YC, Bae EA, Kim IK, et al. Characterization of age-associated exhausted CD8+T cells defined by increased expression of Tim-3 and PD-1. *Aging Cell* (2016) 15:291–300. doi:10.1111/acer.12435
41. Hsu H, Boudova S, Mvula G, Divala TH, Mungwira RG, Harman C, et al. Prolonged PD1 expression on neonatal Vδ2 lymphocytes dampens proinflammatory responses: role of epigenetic regulation. *J Immunol* (2016) 197(5):1884–92. doi:10.4049/jimmunol.1600284
42. De Roock S, Hoeks SBEA, Meurs L, Steur A, Hoekstra MO, Prakken BJ, et al. Critical role for programmed death 1 signaling and protein kinase B in augmented regulatory T-cell induction in cord blood. *J Allergy Clin Immunol* (2011) 128(6):1369–71. doi:10.1016/j.jaci.2011.08.006

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2018 Klocperk, Paračková, Bloomfield, Rataj, Pokorný, Unger, Warnatz and Šedivá. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



Posttranscriptional Gene Regulation of T Follicular Helper Cells by RNA-Binding Proteins and microRNAs

Dirk Baumjohann^{1*} and Vigo Heissmeyer^{1,2*}

¹ Institute for Immunology, Biomedical Center, Ludwig-Maximilians-Universität München, Planegg-Martinsried, Germany,

² Research Unit Molecular Immune Regulation, Helmholtz Zentrum München, Munich, Germany

OPEN ACCESS

Edited by:

Georgia Fousteri,
San Raffaele Hospital (IRCCS), Italy

Reviewed by:

Carolin Daniel,
Helmholtz-Gemeinschaft Deutscher
Forschungszentren (HZ), Germany
Xing Chang,
Shanghai Institutes for Biological
Sciences (CAS), China

*Correspondence:

Dirk Baumjohann
dirk.baumjohann@med.uni-
muenchen.de;
Vigo Heissmeyer
vigo.heissmeyer@med.uni-
muenchen.de

Specialty section:

This article was submitted
to T Cell Biology,
a section of the journal
Frontiers in Immunology

Received: 04 May 2018

Accepted: 20 July 2018

Published: 31 July 2018

Citation:

Baumjohann D and Heissmeyer V
(2018) Posttranscriptional Gene
Regulation of T Follicular Helper
Cells by RNA-Binding Proteins
and microRNAs.
Front. Immunol. 9:1794.
doi: 10.3389/fimmu.2018.01794

T follicular helper (Tfh) cells are critically involved in the establishment of potent antibody responses against infectious pathogens, such as viruses and bacteria, but their dysregulation may also result in aberrant antibody responses that frequently coincide with autoimmune diseases or allergies. The fate and identity of Tfh cells is tightly controlled by gene regulation on the transcriptional and posttranscriptional level. Here, we provide deeper insights into the posttranscriptional mechanisms that regulate Tfh cell differentiation, function, and plasticity through the actions of RNA-binding proteins (RBPs) and small endogenously expressed regulatory RNAs called microRNAs (miRNAs). The Roquin family of RBPs has been shown to dampen spontaneous activation and differentiation of naïve CD4⁺ T cells into Tfh cells, since CD4⁺ T cells with Roquin mutations accumulate as Tfh cells and provide inappropriate B cell help in the production of autoantibodies. Moreover, Regnase-1, an endoribonuclease that regulates a set of targets, which strongly overlaps with that of Roquin, is crucial for the prevention of autoantibody production. Interestingly, both Roquin and Regnase-1 proteins are cleaved and inactivated after TCR stimulation by the paracaspase MALT1. miRNAs are expressed in naïve CD4⁺ T cells and help preventing spontaneous differentiation into effector cells. While most miRNAs are downregulated upon T cell activation, several miRNAs have been shown to regulate the fate of these cells by either promoting (e.g., miR-17-92 and miR-155) or inhibiting (e.g., miR-146a) Tfh cell differentiation. Together, these different aspects highlight a complex and dynamic regulatory network of posttranscriptional gene regulation in Tfh cells that may also be active in other T helper cell populations, including Th1, Th2, Th17, and Treg.

Keywords: T follicular helper, T follicular regulatory, Roquin, regnase-1, microRNAs, miR-17-92, miR-155, miR-146a

INTRODUCTION

T helper cells are important constituents of the adaptive immune system. They are critically involved in the elimination of various pathogens, including viruses, bacteria, and fungi. Due to their capabilities of forming immunological memory and providing help to B cells, vaccines aim at inducing strong T helper cell responses in concert with cytotoxic T cells and antibody-producing

B cells. However, dysregulated T helper cell responses are also associated with several diseases. In allergies, the immune system reacts to normally innocuous compounds of the environment. In autoimmune diseases such as type I diabetes, multiple sclerosis, and rheumatoid arthritis, T helper cells are coordinating critical processes that contribute to tissue inflammation and destruction. In cancer, dysregulated T helper cells might on the one hand be impaired in their proper functioning, thus limiting the body's immune response against neoplastic cells and tissues. On the other hand, hyper-responsiveness or malignant transformation of T helper cells can drive chronic inflammation or induce neoplasia, respectively.

T helper cells comprise many different subsets that are each tailored to the functional response that these cells elicit against various different pathogens. The major T helper cell subsets include Th1, Th2, Th17, T follicular helper (Tfh), and regulatory T (Treg) cells, which can be differentiated by their characteristic expression of signature transcription factors, chemokine receptors, and cytokines (1). While initially it was believed that Th2 cells provide help to B cells, it is now accepted that Tfh cells are the major subset of T helper cells that is specialized in providing help to B cells for the establishment and maintenance of germinal centers (GCs) and for the production of high-affinity antibodies (2–4). In line with this, Tfh cells express the chemokine receptor CXCR5, which facilitates the migration of activated CD4⁺ T cells to the T-B zone border and further into the B cell follicle. This aspect also reflects the step-wise differentiation process of Tfh cells, which are initially primed by dendritic cells, followed by sequential interactions with activated B cells and GC B cells (2–4). Tfh cells produce various cytokines, including IL-21 and IL-4, and they express several costimulatory molecules, including ICOS, CD40L, and PD-1, which allow for reciprocal interactions with B cells. Tfh cells are further characterized by the expression of the transcription factors *Ascl2* and *Bcl6*, and the adaptor molecule SAP. Besides Tfh cells, T follicular regulatory (Tfr) cells have been identified as a hybrid cell population of Tfh and Treg cells that prevent excessive humoral immune responses (5). They express the signature transcription factors of Tfh and Treg cells, *Bcl6* and *Foxp3*, respectively, and share additional characteristics of both T helper cell subsets.

The differentiation of naïve CD4⁺ T cells into effector and memory cells is tightly regulated on the molecular level (6–8). Several signature or “master” transcription factors have been identified that are specific for the respective T helper cell subset, e.g., T-bet for Th1, Gata-3 for Th2, RORγt for Th17, *Bcl6* for Tfh, and *Foxp3* for Treg. Often, these transcription factors also inhibit each other's function, thus contributing to cell fate decisions of the differentiating cells. Upstream of these “lineage”-defining transcription factors, combinations of Jak (Janus kinase) and signal transducer and activator of transcription (STAT) molecules that transduce signaling events from cytokine receptors have also been associated with the different T helper cell populations (9). Given the variety of T helper cell qualities, gene expression needs to be thoroughly regulated in activated CD4⁺ T cells to ensure proper differentiation into the different T helper cell subsets (10). Beside the direct transcriptional regulation through STATs and other transcription factors, transcribed mRNAs are

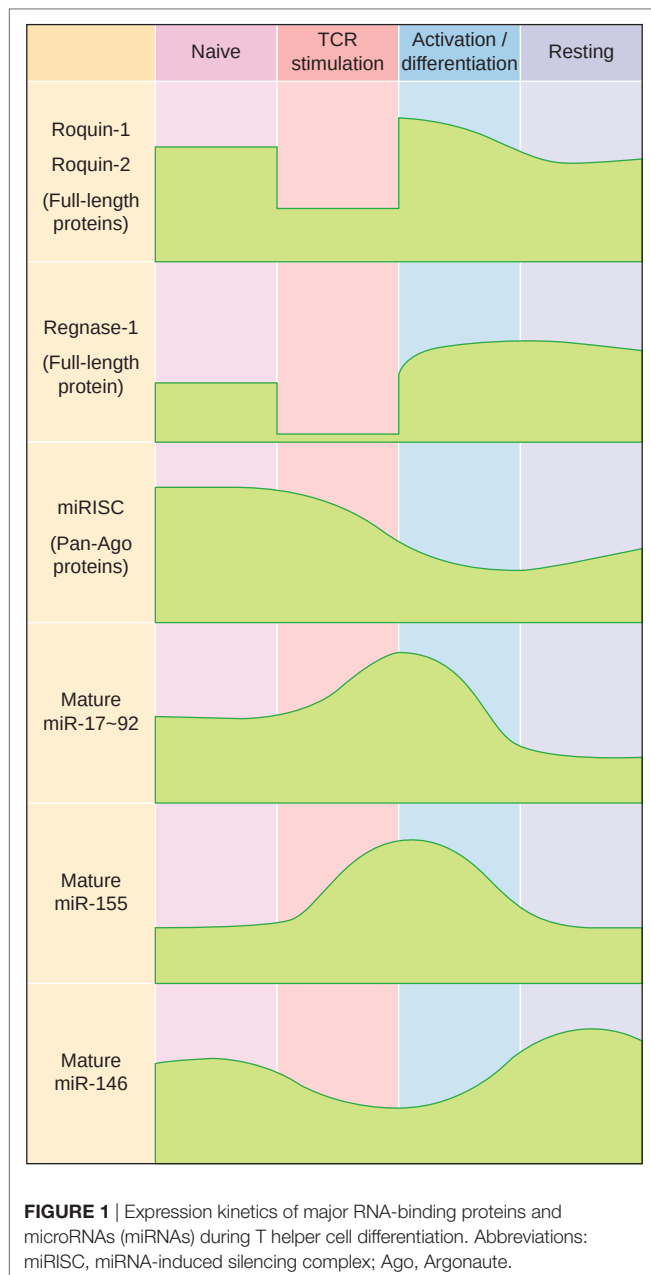
furthermore highly regulated on the posttranscriptional level. Several mechanisms contribute to this regulation, including RNA-binding proteins (RBPs) and microRNAs (miRNAs), which can act cooperatively on different as well as on similar molecular pathways. In this review, we discuss the role of different RBPs and miRNAs in shaping Tfh cell identity and function.

POSTTRANSCRIPTIONAL GENE REGULATION BY RBPs IN T CELLS

RNA-binding proteins are *trans*-acting factors that interact with specific *cis*-elements in RNAs by recognizing linear sequence motifs or dynamically forming secondary structures. The binding to *cis*-elements in the 5' UTR typically controls translation initiation, while binding to sites in the 3' UTRs of transcripts typically regulates mRNA decay or translation efficiency (11).

The Roquin family of RBPs includes the paralogs Roquin-1 and Roquin-2. These proteins are encoded by the *Rc3h1* and *Rc3h2* genes and serve redundant functions in T cells (12–14). The Regnase family comprises the paralogs Regnase-1, Regnase-2, Regnase-3, and Regnase-4 also known as *Mcpi1*, 2, 3, and 4, which are encoded by the *Zc3h12a*, *Zc3h12b*, *Zc3h12c*, and *Zc3h12d* genes (15). The redundancy of Regnase proteins has not been addressed experimentally; however, Regnase-1 and Regnase-4 proteins appear to be the T cell-expressed paralogs (15). Regnase-1 and Roquin proteins predominantly bind to 3' UTRs of mRNAs (16, 17) and play important roles in the regulation of T cell fate decisions (14, 18–22). Roquin proteins recognize stem-loop structures of the tri- or hexa-loop containing CDE or ADE consensus motifs, respectively (17, 23–30). These interactions allow the recruitment of mRNA degrading enzymes (24, 31, 32) and induce decay of target mRNAs. Regnase-1 also appears to repress targets through similar stem-loop structures (16, 21, 33, 34) that are present in an overlapping set of target mRNAs with pro-inflammatory functions (16, 20). However, the endonuclease Regnase-1 may rather cleave target mRNAs itself or, dependent on the 3' UTR, induce translational inhibition (16, 21, 33–35). Among the well-established targets of Roquin and Regnase proteins are *ICOS*, *Ox40*, *Il6*, *cRel*, *Irf4*, *Nfkbiz*, and *Nfkbid* (14, 16–24, 28, 33, 34). Interestingly, the mRNAs encoding for Roquin and Regnase proteins themselves contain *cis*-elements that enable the system to fine-tune expression levels through negative autoregulation (18, 20, 24, 27, 33). However, it is currently under debate whether these factors cooperate in posttranscriptional gene regulation or work independently in a spatially and temporally compartmentalized fashion (16, 20, 36, 37).

While Roquin-1 and the less abundant Roquin-2 proteins show rather constitutive expression in T cells (14) and are only moderately upregulated in response to TCR-dependent T cell stimulation (38), the most prominent member of the Regnase family of proteins in T cells, Regnase-1, is weakly expressed in naïve T cells, but becomes induced during TCR-dependent activation of T cells (39) (Figure 1). However, during TCR signaling itself, Roquin-1 and Roquin-2 as well as Regnase-1 proteins are



cleaved and functionally inactivated by the MALT1 paracaspase (20, 21, 40) (**Figure 1**).

RNA-BINDING PROTEIN-MEDIATED REGULATION OF Tfh AND Tfr CELLS

The Roquin and Regnase-1 RBPs have been shown to be involved in the regulation of the GC reaction and prevention of autoimmunity, since mutation or loss-of-function of the encoding genes lead to spontaneous activation of T cells and the development of antinuclear antibodies in mice (14, 18, 20–22). Their role in Tfh and Tfr cells will be described in more detail here (**Figure 2**).

ROQUIN

The gene encoding for Roquin-1 was identified in the lab of Christopher Goodnow by screening of mice for ethyl nitroso urea-induced mutations that caused the formation of antinuclear autoantibodies (22). Homozygous mutation exchanging one single amino acid of M199R in Roquin-1, as determined in the so-called *sanroque* mouse strain, was found to cause a dramatic activation of CD8⁺ and CD4⁺ T cells and led to the accumulation of Tfh cells. Splens of these mice contained large numbers of GCs and the induced GC B cells produced high-affinity antibodies against a large variety of self-antigens (22, 41). Surprisingly, the knockout of the Roquin-1-encoding gene *Rc3h1* showed postnatal lethality and mild immune dysregulation but did not recapitulate the flagrant autoimmune phenotype of *sanroque* mice (42). Nevertheless, combined deletion of Roquin-1 and Roquin-2 encoding genes in T cells resulted in the spontaneous activation of CD4⁺ and CD8⁺ T cells and the accumulation of Tfh cells and GC B cells. These findings demonstrated redundant functions of both proteins in T cells and suggested a compensatory function of the much lower expressed Roquin-2 protein in the absence of Roquin-1, but not when Roquin-1^{san} protein is expressed (14). In mice lacking Roquin-1 and Roquin-2-encoding alleles in T cells, the splenic architecture was greatly disturbed and, as a probable consequence, less self-reactive antibodies were observed in the sera (14, 20).

The molecular mechanisms underlying spontaneous T cell activation and Tfh cell differentiation are likely to involve several Roquin-regulated targets that synergize in this differentiation program. Initially, the dysregulation of ICOS, the first and best-studied Roquin target (22, 28, 31, 38, 43, 44), was proposed to explain the observed autoimmune phenotype (45). However, *sanroque* mice that were additionally deficient in *Icos* were later shown to maintain many phenotypes including Tfh cell accumulation (46). Instead, accumulation of Tfh cells in *sanroque* mice was a consequence of the excessive production of IFN- γ that occurs in these mice, as was demonstrated in combination of *sanroque* and IFN- γ receptor (*Ifngr*) knockout genotypes (46). At this point, it is not clear how IFN- γ becomes induced in *sanroque* mice, since the *Ifng* mRNA is rather strongly regulated by AU-rich elements (AREs), which are recognized by ARE-binding proteins like TTP, AUF, or HUR proteins, and genetic deletion of these AREs has been demonstrated to also cause a lupus-like phenotype in mice (47, 48). As compared to *sanroque* mice, CD4⁺ T cells lacking Roquin proteins also did not show a similarly strong Th1 bias, but rather differentiated into Th17 cells *in vitro*, a phenotype that developed in addition to the shared spontaneous differentiation into Tfh cells (20). This differential bias may relate to a partial or complete derepression of the different Roquin-regulated targets including *ICOS*, *Irf4*, *cRel*, *Nfkbiz*, and *Nfkbid* that have been shown to affect Tfh as well as Th17 differentiation (49–58). One key signaling cascade influenced by Roquin has been identified in the PI3K-Akt-mTOR and Foxo1 pathway in which Roquin regulates the expression of *ICOS*, *Pten*, and *Itch* mRNAs (19, 31, 44) (**Figure 2**). The *ICOS* and *Itch* mRNAs are bound and negatively regulated, leading to increased *ICOS* and *Itch* levels in the absence of Roquin (19, 28, 31, 38). Increased *ICOS* expression

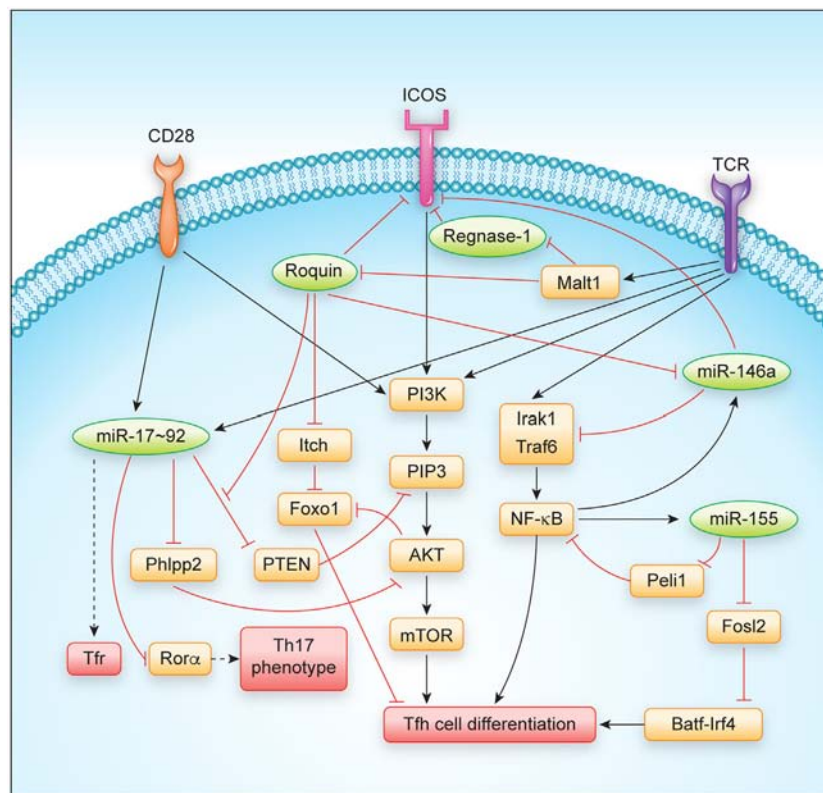


FIGURE 2 | Regulation of T follicular helper cell differentiation and function by major RNA-binding proteins and microRNAs. Abbreviations: TCR, T cell receptor.

and signaling stimulates PIP3 formation that activates the kinase Akt, which phosphorylates and thereby inactivates Foxo1, a transcription factor that strongly inhibits Tfh differentiation (57). In contrast, elevation of Itch, a Foxo1-specific E3 ubiquitin ligase, decreases cellular levels of Foxo1 (57, 59). Different from the other Roquin targets, Pten levels decreased in CD4⁺ T cells and Treg cells upon induced ablation of Roquin-encoding alleles (19). Interestingly, the Roquin-bound sequences in the 3' UTR of *Pten* showed conservation of those nucleotides that were involved in forming a stem-loop (19), but at the same time overlapped with a miR-17 binding site, which was previously shown to effectively regulate Pten levels in T cells (60). Biochemical evidence showed that Ago2 more efficiently associated with *Pten* mRNA in the absence of Roquin (19), suggesting a structure switch mode within this *cis*-element and a competitive interaction and regulation of *Pten* mRNA by Roquin and miR-17-92 containing RNA-induced silencing complex (miRISC) transacting factors. The regulation of PI3K-Akt-mTOR and Foxo1 signaling through Roquin-mediated regulation of ICOS, Itch, and Pten targets not only contributes to the observed skewing of T cell differentiation into Tfh and Th17 and against induced Treg (iTreg) cell differentiation, but also correlates with a conversion of thymus-derived Treg cells into Tfr cells *in vivo* (19). Roquin-deficient Treg cells lost CD25 expression, upregulated a Tfh but downregulated their Treg gene signature and retained their ability to control antigen-dependent GC B cell responses and affinity maturation

of antibodies. In contrast, Roquin-deficient Treg cells were less able to prevent spontaneous activation of CD4⁺ and CD8⁺ T cells and to protect from T cell transfer-induced colitis (19).

REGNASE-1

Regnase-1 was initially described as an LPS-induced gene and the knockout of Regnase-1 caused a severe auto-inflammatory phenotype in mice. The molecular basis for this phenotype was proposed to involve Regnase-1-dependent regulation of IL-6 and IL-12p40 regulation in myeloid cells (34). However, more recently, it was shown that the combined knockout of Regnase-1 with IL-6 or IL-12-encoding alleles did not fully rescue central phenotypes. Instead, conditional T cell-specific deletion of Regnase-1 phenocopied most of the phenotypes of the global Regnase-1 knockout (21). The consequences of Regnase-1 deficiency for Tfh differentiation have not been experimentally addressed so far, but the following observations could argue for a control of Tfh differentiation by Regnase-1: First, upon genetic inactivation of Regnase-1 globally or specifically in T cells, mice develop autoantibodies and show elevated plasma cell levels as well as an accumulation of all immunoglobulin isotypes in their sera (21, 34). Second, at least for the regulation of a CDE-containing element in the 3' UTR of the *Tnf* mRNA, Regnase-1 has been demonstrated to functionally cooperate with Roquin in target regulation, and this mode of direct or indirect interaction may apply to several other shared

target mRNAs that have an effect on Tfh cell differentiation (20, 37). Moreover, the systemic IFN- γ production that was found to drive Tfh cell differentiation in *sanroque* mice (46) was similarly observed upon deletion of Regnase-1-encoding alleles in T cells (18, 21). Finally, among the targets that have been reported to be regulated by Regnase-1 are several gene products that are known to promote Tfh differentiation, including ICOS, Ox40, and IL-6 (2, 61). Future experiments should demonstrate how Regnase-1 or other Regnase paralogs affect Tfh cell differentiation in a T cell-intrinsic or extrinsic manner.

POSTTRANSCRIPTIONAL GENE REGULATION BY miRNAs IN T CELLS

MicroRNAs are small endogenously expressed RNAs that regulate gene expression. Each miRNA can have several hundred target genes and a given mRNA might in turn be regulated by many different miRNAs simultaneously. These features of miRNAs result in redundancy that is believed to buffer gene expression and to confer biological robustness (62–64). In line with this, miRNAs and the miRNA-induced silencing complex (miRISC) are relatively highly expressed in naïve CD4⁺ T cells, thereby contributing to the prevention of spontaneous differentiation into effector cells (65, 66) (**Figure 1**). In activated T cells, most miRNAs are downregulated, while only a few so-called driver-miRNAs are differentially upregulated to act in concert with transcription factors for proper T helper cell differentiation and function (67–71) (**Figure 1**). Initial experiments that utilized genetically engineered mice in which T cells lacked mature miRNAs due to ablation of miRNA-processing proteins such as Dicer established functional roles for miRNAs in the generation of Th1, Th2, Th17, and Treg cells (72, 73). While all these T helper cell populations could still be generated to various degrees from miRNA-deficient naïve CD4⁺ precursor cells, global miRNA expression in CD4⁺ T cells was absolutely required for the differentiation of naïve CD4⁺ T cells into mature Tfh cells *in vivo* (74). To date, various T cell-expressed miRNAs and miRNA clusters have been shown to play critical roles in the differentiation and function of Tfh cells (69) and in the establishment and maintenance of GCs (75), and these findings indicate that Tfh cells may be particularly sensitive to the regulation by miRNAs.

miRNA-MEDIATED REGULATION OF Tfh AND Tfr CELLS

Among the individual miRNAs that have been studied in the context of Tfh cells, the function of the miR-17–92 cluster and the miR-155/miR-146a axis have been investigated in most detail and will be described here (**Figure 2**).

THE miR-17–92 CLUSTER

The miR-17–92 cluster consists of six individual miRNAs that can be grouped into four distinct miRNA families according to their seed sequences (76). Even though miR-17–92 is transcribed as a common transcript and highly induced in activated CD4⁺ T cells

(65, 77–80) (**Figure 1**), the individual miRNA cluster members are differentially processed thereafter (81). miR-17–92 is not only critically involved in the regulation of Tfh cells (as discussed in more detail below), but it is also important for the differentiation and function of other T helper cell subsets [reviewed in Ref. (82)], including Th1 (80, 83), Treg (77, 83), Th2 (84), and Th17 cells (79, 85). Interestingly, miR-17–92 shares several features between Th2 cells and type 2 innate lymphoid cells (86), indicating that many of the miR-17–92 cluster's functions may be conserved between the individual T helper cell subsets and their respective ILC counterparts. First evidence for a role of miR-17–92 in Tfh cells (**Figure 1**) came from an early study in which Bcl6 overexpression resulted in reduced miR-17–92 expression, with miR-17–92 itself repressing CXCR5 (87). However, more recent studies clarified the Tfh-promoting function of miR-17–92 (74, 78, 80). Deletion of the miR-17–92 cluster in T cells resulted in reduced Tfh cell differentiation, whereas transgenic overexpression of the cluster resulted in higher frequencies and numbers of Tfh cells. On the mechanistic level, miR-17–92 was found to target *Pten* and *Phlpp2*, a phosphatase in the ICOS signaling pathway (74, 78). Besides these Tfh-promoting effects, miR-17–92 also prevented the expression of genes that are normally not associated with Tfh cells during LCMV infection, but instead are usually associated with Th17 cells, including *Ccr6*, *Rora*, *Il22*, *Il1r1*, and *Il1r2* (74). Importantly, it was further shown that each miRNA of the miR-17–92 cluster directly targeted the *Rora* 3' UTR and that this axis contributed to repressing the Th17-associated gene expression program in wild-type Tfh cells (74). Since most of these experiments were performed with mice deficient in or overexpressing the entire miR-17–92 cluster in T cells *in vivo*, not much is known about the contribution of the individual miRNAs of this cluster to Tfh cell differentiation and function. This would be important though, because individual miRNAs of this cluster can have cooperative but also opposing effects on T cells, which is further amplified by the complexity of the downstream target gene networks. The continuing lack of reliable protocols for the *in vitro* differentiation of murine Tfh cells (2) currently impairs the ability to perform *in vitro* experiments that interrogate individual miRNA functions specifically in mouse Tfh cells. Nevertheless, recent technological advances such as CRISPR/Cas9 or mouse lines that lack individual cluster members (88) might substitute for this current limitation. Using human Tfh cell *in vitro* cultures, a recent report found that miR-92a targets *KLF2* and *PTEN*, thereby promoting Tfh cell differentiation (89). Similar to Tfh cells, Tfr cells are also responsive to the dose of miR-17–92 regulation (74). As a first example of how RBPs can intersect with miRNA-dependent gene regulation of Tfh differentiation, it was recently shown that Roquin (see above) interferes with miR-17–92 binding to an overlapping *cis*-element in the *Pten* 3' UTR, which leads to inhibition of the PI3K–Akt–mTOR signaling pathway, thereby inhibiting the conversion of Treg to Tfr cells (19).

THE miR-155/miR-146a AXIS

Similar to miR-17–92, miR-155 is induced and highly expressed in activated T helper cells (65, 90–93) (**Figure 1**). miR-155 has

been shown to be important for proper differentiation of Th1 and Th17 cells and for EAE pathogenesis (94–96), as well as for Treg differentiation and function (97, 98). In contrast to miR-155, miR-146a is highly expressed in naïve CD4⁺ T cells and initially downregulated in activated T cells (65, 90) (**Figure 1**). miR-146a is subsequently upregulated in differentiating Tfh cells (55), reaching the highest expression levels among hematopoietic cells in mature Tfh and GC B cells (99). In comparison to the total numbers of Tfh cells elicited during an immune response, the kinetics of increased miR-146a expression slightly lagged behind (55). These data indicate that miR-146a acts as a negative regulator of Tfh cells that prevents excessive Tfh cell numbers and thereby limits GC responses (69). In T cells, an epistatic relationship between miR-146a and miR-155 has been described in which miR-155 promotes and miR-146 inhibits IFN γ responses (100). Further studies have also established a role for miR-146a in T cell activation (101, 102) as well as in differentiation and function of different T helper cell subsets, including Th1, Th17, and Treg cells (103–106). miR-146a-deficient mice develop a chronic inflammatory phenotype with progressive myeloproliferation and eventually myeloid and lymphoid malignancies (107). In these mice, Tfh cells accumulate due to the Tfh cell-promoting function of miR-155 (see below), thus further highlighting the reciprocal regulation of Tfh cell differentiation by these two miRNAs (108). Mechanistically, miR-146a was found to repress several Tfh-associated genes, including ICOS, which was highly upregulated in miR-146a-deficient CD4⁺ T cells (55). miR-146a itself is also regulated by Roquin (43). In a different study, it was shown that miR-155 promoted Tfh cell differentiation by repressing the expression of *Peli1*, a ubiquitin ligase that promotes the degradation of the NF- κ B family transcription factor c-Rel, which itself controls cellular proliferation and CD40L expression (109). Another study found that miR-155 expression in hematopoietic cells was required for the differentiation of Tfh and GC B cells following murine gammaherpesvirus infection (110). Together, these data indicate a tightly controlled reciprocal function of miR-155 and miR-146a in the regulation of Tfh cell differentiation and function.

CONCLUSION

T follicular helper cells require continuous stimulation (111, 112) and the differentiation of these cells is more dependent on costimulatory signals than other T helper cell subsets (113). This might be the reason for why they are so responsive to the regulation by RBPs and miRNAs. Both classes of trans-acting factors cooperate to shape the expression levels not only of costimulatory molecules but also of intracellular transducers of signals in the PI3K-Akt-mTOR and Foxo1 pathway (**Figure 2**). PI3K activity is strongly stimulated by ICOS co-stimulation as well as TCR signaling and was shown to drive the Tfh differentiation program (114). Besides the well-established miRNA regulators of Tfh cells, the miR-17–92 cluster and the miR-155/miR-146a

axis, other miRNAs may also play important roles in Tfh cell differentiation and T helper cell plasticity. For example, miR-10a is highly expressed in Treg cells (99, 115, 116). TGF- β induced miR-10a represses the Tfh-associated transcriptional repressor *Bcl6*, thereby preventing the conversion of Treg cells into Tfh cells (116). In human Tfh cells, miR-31 was recently shown to be downregulated by BCL6, thereby resulting in the upregulation of the miR-31 target mRNAs encoding the Tfh-associated molecules CD40L, SAP, and BTLA (117). The finding that Tfh cell differentiation is completely blocked in miRNA-deficient CD4⁺ T cells (74) further indicates that additional miRNAs must play important roles in Tfh cell biology. Moreover, it remains largely unclear in how far miRNAs and their target gene networks contribute to the function and maintenance of Tfh cells. It is also likely that upon comprehensive identification of RBPs that are bound to mRNAs in T cells and upon individual testing of these factors, additional contributions of RBPs in T cell differentiation will be uncovered. Such future investigations will provide novel insights into how the loss of individual miRNAs or individual RBPs affects T cell differentiation programs. Additional contributions may result from other levels of posttranscriptional regulation such as alternative splicing, alternative polyadenylation, or RNA modifications, which have already been shown to regulate Tfh-relevant genes (118–120), albeit, it has not yet been tested how these processes may impact Tfh differentiation. Another big task will be to understand how simultaneous inputs from different posttranscriptional regulators generate a specific response. Here, it will be critical to comprehensively identify and dissect all *cis*-elements encoded in mRNAs with strong impact on T cell differentiation programs (**Figure 2**). These analyses should be combined with structural and biochemical information to integrate the emerging evidence of RNA-modifications, to describe dynamics of RNA/protein-complex formation, and to understand how the individual binding sites act redundantly, cooperatively, antagonistically, or synergistically in posttranscriptional gene regulation, thus enabling cell-fate decisions.

AUTHOR CONTRIBUTIONS

Both authors contributed to conceptualizing and writing the manuscript.

FUNDING

This work was supported by the Deutsche Forschungsgemeinschaft Emmy Noether Programme (BA 5132/1-1) (DB), SFB 1054 Teilprojekt B12 (DB), and SFB 1054 Teilprojekt A03, Z02 (VH), as well as through grants from the Fritz Thyssen Stiftung (10.16.1.021MN) and Else Kröner-Fresenius-Stiftung (2015_A158) (VH). The authors would like to thank Julia Maul, Stephanie Edelmann, and Nina Kronbeck for critical reading of the manuscript.

REFERENCES

1. Sallusto F. Heterogeneity of human CD4(+) T cells against microbes. *Annu Rev Immunol* (2016) 34:317–34. doi:10.1146/annurev-immunol-032414-112056
2. Crotty S. T follicular helper cell differentiation, function, and roles in disease. *Immunity* (2014) 41:529–42. doi:10.1016/j.immuni.2014.10.004
3. Qi H. T follicular helper cells in space-time. *Nat Rev Immunol* (2016) 16(10):612–25. doi:10.1038/nri.2016.94

4. Vinuesa CG, Linterman MA, Yu D, MacLennan IC. Follicular helper T cells. *Annu Rev Immunol* (2016) 34:335–68. doi:10.1146/annurev-immunol-041015-055605
5. Sage PT, Sharpe AH. T follicular regulatory cells. *Immunol Rev* (2016) 271:246–59. doi:10.1111/imr.12411
6. DuPage M, Bluestone JA. Harnessing the plasticity of CD4(+) T cells to treat immune-mediated disease. *Nat Rev Immunol* (2016) 16:149–63. doi:10.1038/nri.2015.18
7. O'Shea JJ, Paul WE. Mechanisms underlying lineage commitment and plasticity of helper CD4+ T cells. *Science* (2010) 327:1098–102. doi:10.1126/science.1178334
8. Zhu J, Yamane H, Paul WE. Differentiation of effector CD4 T cell populations (*). *Annu Rev Immunol* (2010) 28:445–89. doi:10.1146/annurev-immunol-030409-101212
9. O'Shea JJ, Schwartz DM, Villarino AV, Gadina M, McInnes IB, Laurence A. The JAK-STAT pathway: impact on human disease and therapeutic intervention. *Annu Rev Med* (2015) 66:311–28. doi:10.1146/annurev-med-051113-024537
10. Fang D, Zhu J. Dynamic balance between master transcription factors determines the fates and functions of CD4 T cell and innate lymphoid cell subsets. *J Exp Med* (2017) 214:1861–76. doi:10.1084/jem.20170494
11. Rissland OS. The organization and regulation of mRNA-protein complexes. *Wiley Interdiscip Rev RNA* (2017) 8(1):e1369. doi:10.1002/wrna.1369
12. Heissmeyer V, Vogel KU. Molecular control of Tfh-cell differentiation by Roquin family proteins. *Immunol Rev* (2013) 253:273–89. doi:10.1111/imr.12056
13. Pratama A, Ramiscal RR, Silva DG, Das SK, Athanasopoulos V, Fitch J, et al. Roquin-2 shares functions with its paralog Roquin-1 in the repression of mRNAs controlling T follicular helper cells and systemic inflammation. *Immunity* (2013) 38:669–80. doi:10.1016/j.immuni.2013.01.011
14. Vogel KU, Edelmann SL, Jeltsch KM, Bertossi A, Heger K, Heinz GA, et al. Roquin paralogs 1 and 2 redundantly repress the Icos and Ox40 costimulator mRNAs and control follicular helper T cell differentiation. *Immunity* (2013) 38:655–68. doi:10.1016/j.immuni.2012.12.004
15. Akira S. Regnase-1, a ribonuclease involved in the regulation of immune responses. *Cold Spring Harb Symp Quant Biol* (2013) 78:51–60. doi:10.1101/sqb.2013.78.019877
16. Mino T, Murakawa Y, Fukao A, Vandenbon A, Wessels HH, Ori D, et al. Regnase-1 and Roquin regulate a common element in inflammatory mRNAs by spatiotemporally distinct mechanisms. *Cell* (2015) 161:1058–73. doi:10.1016/j.cell.2015.04.029
17. Murakawa Y, Hinz M, Mothes J, Schuetz A, Uhl M, Wyler E, et al. RC3H1 post-transcriptionally regulates A20 mRNA and modulates the activity of the IKK/NF-kappaB pathway. *Nat Commun* (2015) 6:7367. doi:10.1038/ncomms8367
18. Cui X, Mino T, Yoshinaga M, Nakatsuka Y, Hia F, Yamasoba D, et al. Regnase-1 and Roquin nonredundantly regulate Th1 differentiation causing cardiac inflammation and fibrosis. *J Immunol* (2017) 199:4066–77. doi:10.4049/jimmunol.1701211
19. Essig K, Hu D, Guimaraes JC, Alterauge D, Edelmann S, Raj T, et al. Roquin suppresses the PI3K-mTOR signaling pathway to inhibit T helper cell differentiation and conversion of Treg to Tfr cells. *Immunity* (2017) 47:1067–1082. doi:10.1016/j.immuni.2017.11.008
20. Jeltsch KM, Hu D, Brenner S, Zoller J, Heinz GA, Nagel D, et al. Cleavage of roquin and regnase-1 by the paracaspase MALT1 releases their cooperatively repressed targets to promote T(H)17 differentiation. *Nat Immunol* (2014) 15:1079–89. doi:10.1038/ni.3008
21. Uehata T, Iwasaki H, Vandenbon A, Matsushita K, Hernandez-Cuellar E, Kuniyoshi K, et al. Malt1-induced cleavage of regnase-1 in CD4(+) helper T cells regulates immune activation. *Cell* (2013) 153:1036–49. doi:10.1016/j.cell.2013.04.034
22. Vinuesa CG, Cook MC, Angelucci C, Athanasopoulos V, Rui L, Hill KM, et al. A RING-type ubiquitin ligase family member required to repress follicular helper T cells and autoimmunity. *Nature* (2005) 435:452–8. doi:10.1038/nature03555
23. Janowski R, Heinz GA, Schlundt A, Wommelsdorf N, Brenner S, Gruber AR, et al. Roquin recognizes a non-canonical hexaloop structure in the 3'-UTR of Ox40. *Nat Commun* (2016) 7:11032. doi:10.1038/ncomms11032
24. Leppek K, Schott J, Reitter S, Poetz F, Hammond MC, Stoecklin G. Roquin promotes constitutive mRNA decay via a conserved class of stem-loop recognition motifs. *Cell* (2013) 153:869–81. doi:10.1016/j.cell.2013.04.016
25. Oikonomou P, Goodarzi H, Tavazoie S. Systematic identification of regulatory elements in conserved 3' UTRs of human transcripts. *Cell Rep* (2014) 7:281–92. doi:10.1016/j.celrep.2014.03.001
26. Ramanathan M, Majzoub K, Rao DS, Neela PH, Zarnegar BJ, Mondal S, et al. RNA-protein interaction detection in living cells. *Nat Methods* (2018) 15:207–12. doi:10.1038/nmeth.4601
27. Sakurai S, Ohto U, Shimizu T. Structure of human Roquin-2 and its complex with constitutive-decay element RNA. *Acta Crystallogr F Struct Biol Commun* (2015) 71:1048–54. doi:10.1107/S2053230X15011887
28. Schlundt A, Heinz GA, Janowski R, Geerloff A, Stehle R, Heissmeyer V, et al. Structural basis for RNA recognition in roquin-mediated post-transcriptional gene regulation. *Nat Struct Mol Biol* (2014) 21:671–8. doi:10.1038/nsmb.2855
29. Schuetz A, Murakawa Y, Rosenbaum E, Landthaler M, Heinemann U. Roquin binding to target mRNAs involves a winged helix-turn-helix motif. *Nat Commun* (2014) 5:5701. doi:10.1038/ncomms6701
30. Tan D, Zhou M, Kiledjian M, Tong L. The ROQ domain of Roquin recognizes mRNA constitutive-decay element and double-stranded RNA. *Nat Struct Mol Biol* (2014) 21:679–85. doi:10.1038/nsmb.2857
31. Glasmacher E, Hoefig KP, Vogel KU, Rath N, Du L, Wolf C, et al. Roquin binds inducible costimulator mRNA and effectors of mRNA decay to induce microRNA-independent post-transcriptional repression. *Nat Immunol* (2010) 11:725–33. doi:10.1038/ni.1902
32. Sgromo A, Raich T, Bawankar P, Bhandari D, Chen Y, Kuzuoglu-Ozturk D, et al. A CAF40-binding motif facilitates recruitment of the CCR4-NOT complex to mRNAs targeted by *Drosophila* Roquin. *Nat Commun* (2017) 8:14307. doi:10.1038/ncomms14307
33. Iwasaki H, Takeuchi O, Teraguchi S, Matsushita K, Uehata T, Kuniyoshi K, et al. The IkappaB kinase complex regulates the stability of cytokine-encoding mRNA induced by TLR-IL-1R by controlling degradation of regnase-1. *Nat Immunol* (2011) 12:1167–75. doi:10.1038/ni.2137
34. Matsushita K, Takeuchi O, Standley DM, Kumagai Y, Kawagoe T, Miyake T, et al. Zc3h12a is an RNase essential for controlling immune responses by regulating mRNA decay. *Nature* (2009) 458:1185–90. doi:10.1038/nature07924
35. Behrens G, Winzen R, Rehage N, Dorrie A, Barsch M, Hoffmann A, et al. A translational silencing function of MCP1P/Regnase-1 specified by the target site context. *Nucleic Acids Res* (2018) 46:4256–70. doi:10.1093/nar/gky106
36. Fu M, Blackshear PJ. RNA-binding proteins in immune regulation: a focus on CCCH zinc finger proteins. *Nat Rev Immunol* (2017) 17:130–43. doi:10.1038/nri.2016.129
37. Jeltsch KM, Heissmeyer V. Regulation of T cell signaling and autoimmunity by RNA-binding proteins. *Curr Opin Immunol* (2016) 39:127–35. doi:10.1016/j.coi.2016.01.011
38. Rehage N, Davydova E, Conrad C, Behrens G, Maiser A, Stehlein JE, et al. Binding of NUFIP2 to Roquin promotes recognition and regulation of ICOS mRNA. *Nat Commun* (2018) 9:299. doi:10.1038/s41467-017-02582-1
39. Li M, Cao W, Liu H, Zhang W, Liu X, Cai Z, et al. MCP1P down-regulates IL-2 expression through an ARE-independent pathway. *PLoS One* (2012) 7:e49841. doi:10.1371/journal.pone.0049841
40. Gewies A, Gorka O, Bergmann H, Pechloff K, Petermann F, Jeltsch KM, et al. Uncoupling Malt1 threshold function from paracaspase activity results in destructive autoimmune inflammation. *Cell Rep* (2014) 9:1292–305. doi:10.1016/j.celrep.2014.10.044
41. Linterman MA, Rigby RJ, Wong RK, Yu D, Brink R, Cannons JL, et al. Follicular helper T cells are required for systemic autoimmunity. *J Exp Med* (2009b) 206:561–76. doi:10.1084/jem.20081886
42. Bertossi A, Aichinger M, Sansonetti P, Lech M, Neff F, Pal M, et al. Loss of Roquin induces early death and immune deregulation but not autoimmunity. *J Exp Med* (2011) 208:1749–56. doi:10.1084/jem.20110578
43. Srivastava M, Duan G, Kershaw NJ, Athanasopoulos V, Yeo JH, Ose T, et al. Roquin binds microRNA-146a and Argonaute2 to regulate microRNA homeostasis. *Nat Commun* (2015) 6:6253. doi:10.1038/ncomms7253
44. Yu D, Tan AH, Hu X, Athanasopoulos V, Simpson N, Silva DG, et al. Roquin represses autoimmunity by limiting inducible T-cell co-stimulator messenger RNA. *Nature* (2007) 450:299–303. doi:10.1038/nature06253

45. Linterman MA, Rigby RJ, Wong R, Silva D, Withers D, Anderson G, et al. Roquin differentiates the specialized functions of duplicated T cell costimulatory receptor genes CD28 and ICOS. *Immunity* (2009) 30:228–41. doi:10.1016/j.immuni.2008.12.015
46. Lee SK, Silva DG, Martin JL, Pratama A, Hu X, Chang PP, et al. Interferon-gamma excess leads to pathogenic accumulation of follicular helper T cells and germinal centers. *Immunity* (2012) 37:880–92. doi:10.1016/j.immuni.2012.10.010
47. Hodge DL, Berthet C, Coppola V, Kastenmuller W, Buschman MD, Schaughency PM, et al. IFN-gamma AU-rich element removal promotes chronic IFN-gamma expression and autoimmunity in mice. *J Autoimmun* (2014) 53:33–45. doi:10.1016/j.jaut.2014.02.003
48. Villarino AV, Katzman SD, Gallo E, Miller O, Jiang S, McManus MT, et al. Posttranscriptional silencing of effector cytokine mRNA underlies the anergic phenotype of self-reactive T cells. *Immunity* (2011) 34:50–60. doi:10.1016/j.immuni.2010.12.014
49. Annemann M, Wang Z, Plaza-Sirvent C, Glauben R, Schuster M, Ewald Sander F, et al. IkappaBNS regulates murine Th17 differentiation during gut inflammation and infection. *J Immunol* (2015) 194:2888–98. doi:10.4049/jimmunol.1401964
50. Brustle A, Heink S, Huber M, Rosenplanter C, Stadelmann C, Yu P, et al. The development of inflammatory T(H)-17 cells requires interferon-regulatory factor 4. *Nat Immunol* (2007) 8:958–66. doi:10.1038/ni1500
51. Chen G, Hardy K, Pagler E, Ma L, Lee S, Gerondakis S, et al. The NF-kappaB transcription factor c-Rel is required for Th17 effector cell development in experimental autoimmune encephalomyelitis. *J Immunol* (2011) 187:4483–91. doi:10.4049/jimmunol.1101757
52. Dong C, Temann UA, Flavell RA. Cutting edge: critical role of inducible costimulator in germinal center reactions. *J Immunol* (2001) 166:3659–62. doi:10.4049/jimmunol.166.6.3659
53. Kobayashi S, Hara A, Isagawa T, Manabe I, Takeda K, MaruYama T. The nuclear IkappaB family protein IkappaBNS influences the susceptibility to experimental autoimmune encephalomyelitis in a murine model. *PLoS One* (2014) 9:e110838. doi:10.1371/journal.pone.0110838
54. Okamoto K, Iwai Y, Oh-Hora M, Yamamoto M, Morio T, Aoki K, et al. IkappaBzeta regulates T(H)17 development by cooperating with ROR nuclear receptors. *Nature* (2010) 464:1381–5. doi:10.1038/nature08922
55. Pratama A, Srivastava M, Williams NJ, Papa I, Lee SK, Dinh XT, et al. MicroRNA-146a regulates ICOS-ICOSL signalling to limit accumulation of T follicular helper cells and germinal centres. *Nat Commun* (2015) 6:6436. doi:10.1038/ncomms7436
56. Ruan Q, Kameswaran V, Zhang Y, Zheng S, Sun J, Wang J, et al. The Th17 immune response is controlled by the Rel-RORgamma-RORgamma T transcriptional axis. *J Exp Med* (2011) 208:2321–33. doi:10.1084/jem.20110462
57. Stone EL, Pepper M, Katayama CD, Kerdiles YM, Lai CY, Emslie E, et al. ICOS coreceptor signaling inactivates the transcription factor FOXO1 to promote Th cell differentiation. *Immunity* (2015) 42:239–51. doi:10.1016/j.immuni.2015.01.017
58. Warnatz K, Bossaller L, Salzer U, Skrabl-Baumgartner A, Schwinger W, van der Burg M, et al. Human ICOS deficiency abrogates the germinal center reaction and provides a monogenic model for common variable immunodeficiency. *Blood* (2006) 107:3045–52. doi:10.1182/blood-2005-07-2955
59. Xiao N, Eto D, Elly C, Peng G, Crotty S, Liu YC. The E3 ubiquitin ligase Itch is required for the differentiation of follicular helper T cells. *Nat Immunol* (2014) 15:657–66. doi:10.1038/ni.2912
60. Xiao C, Srinivasan L, Calado DP, Patterson HC, Zhang B, Wang J, et al. Lymphoproliferative disease and autoimmunity in mice with increased miR-17-92 expression in lymphocytes. *Nat Immunol* (2008) 9:405–14. doi:10.1038/ni1575
61. Tahiliani V, Hutchinson TE, Abboud G, Croft M, Salek-Ardakani S. OX40 cooperates with ICOS To amplify follicular Th cell development and germinal center reactions during infection. *J Immunol* (2017) 198:218–28. doi:10.4049/jimmunol.1601356
62. Bartel DP. MicroRNAs: target recognition and regulatory functions. *Cell* (2009) 136:215–33. doi:10.1016/j.cell.2009.01.002
63. Ebert MS, Sharp PA. Roles for microRNAs in conferring robustness to biological processes. *Cell* (2012) 149:515–24. doi:10.1016/j.cell.2012.04.005
64. Mendell JT, Olson EN. MicroRNAs in stress signaling and human disease. *Cell* (2012) 148:1172–87. doi:10.1016/j.cell.2012.02.005
65. Bronevetsky Y, Villarino AV, Easley CJ, Barbeau R, Barczak AJ, Heinz GA, et al. T cell activation induces proteasomal degradation of Argonaute and rapid remodeling of the microRNA repertoire. *J Exp Med* (2013) 210:417–32. doi:10.1084/jem.20111717
66. Rossi RL, Rossetti G, Wenandy L, Curti S, Ripamonti A, Bonnal RJ, et al. Distinct microRNA signatures in human lymphocyte subsets and enforcement of the naive state in CD4+ T cells by the microRNA miR-125b. *Nat Immunol* (2011) 12:796–803. doi:10.1038/ni.2057
67. Baumjohann D, Ansel KM. MicroRNA-mediated regulation of T helper cell differentiation and plasticity. *Nat Rev Immunol* (2013) 13:666–78. doi:10.1038/nri3494
68. Jeker LT, Bluestone JA. MicroRNA regulation of T-cell differentiation and function. *Immunol Rev* (2013) 253:65–81. doi:10.1111/imr.12061
69. Maul J, Baumjohann D. Emerging roles for microRNAs in T follicular helper cell differentiation. *Trends Immunol* (2016) 37:297–309. doi:10.1016/j.it.2016.03.003
70. Monticelli S. MicroRNAs in T helper cell differentiation and plasticity. *Semin Immunol* (2013) 25:291–8. doi:10.1016/j.smim.2013.10.015
71. Pagani M, Rossetti G, Panzeri I, de Candia P, Bonnal RJ, Rossi RL, et al. Role of microRNAs and long-non-coding RNAs in CD4(+) T-cell differentiation. *Immunol Rev* (2013) 253:82–96. doi:10.1111/imr.12055
72. Cobb BS, Hertweck A, Smith J, O'Connor E, Graf D, Cook T, et al. A role for dicer in immune regulation. *J Exp Med* (2006) 203:2519–27. doi:10.1084/jem.20061692
73. Muljo SA, Ansel KM, Kanellopoulou C, Livingston DM, Rao A, Rajewsky K. Aberrant T cell differentiation in the absence of Dicer. *J Exp Med* (2005) 202:261–9. doi:10.1084/jem.20050678
74. Baumjohann D, Kageyama R, Clingan JM, Morar MM, Patel S, de Kouchkovsky D, et al. The microRNA cluster miR-17 approximately 92 promotes TFH cell differentiation and represses subset-inappropriate gene expression. *Nat Immunol* (2013a) 14:840–8. doi:10.1038/ni.2642
75. Baumjohann D, Ansel KM. MicroRNA regulation of the germinal center response. *Curr Opin Immunol* (2014) 28:6–11. doi:10.1016/j.coi.2014.01.003
76. Mendell JT. miRiad roles for the miR-17-92 cluster in development and disease. *Cell* (2008) 133:217–22. doi:10.1016/j.cell.2008.04.001
77. de Kouchkovsky D, Esensten JH, Rosenthal WL, Morar MM, Bluestone JA, Jeker LT. MicroRNA-17-92 regulates IL-10 production by regulatory T cells and control of experimental autoimmune encephalomyelitis. *J Immunol* (2013) 191:1594–605. doi:10.4049/jimmunol.1203567
78. Kang SG, Liu WH, Lu P, Jin HY, Lim HW, Shepherd J, et al. MicroRNAs of the miR-17 approximately 92 family are critical regulators of TFH differentiation. *Nat Immunol* (2013) 14:849–57. doi:10.1038/ni.2648
79. Montoya MM, Maul J, Singh PB, Pua HH, Dahlstrom F, Wu N, et al. A distinct inhibitory function for miR-18a in Th17 cell differentiation. *J Immunol* (2017) 199:559–69. doi:10.4049/jimmunol.1700170
80. Wu T, Wieland A, Lee J, Hale JS, Han JH, Xu X, et al. Cutting edge: miR-17-92 is required for both CD4 Th1 and T follicular helper cell responses during viral infection. *J Immunol* (2015) 195:2515–9. doi:10.4049/jimmunol.1500317
81. Olive V, Li Q, He L. miR-17-92: a polycistronic oncomir with pleiotropic functions. *Immunol Rev* (2013) 253:158–66. doi:10.1111/imr.12054
82. Baumjohann D. Diverse functions of miR-17-92 cluster microRNAs in T helper cells. *Cancer Lett* (2018) 423:147–52. doi:10.1016/j.canlet.2018.02.035
83. Jiang S, Li C, Olive V, Lykken E, Feng F, Sevilla J, et al. Molecular dissection of the miR-17-92 cluster's critical dual roles in promoting Th1 responses and preventing inducible Treg differentiation. *Blood* (2011) 118:5487–97. doi:10.1182/blood-2011-05-355644
84. Simpson LJ, Patel S, Bhakta NR, Choy DF, Brightbill HD, Ren X, et al. A microRNA upregulated in asthma airway T cells promotes TH2 cytokine production. *Nat Immunol* (2014) 15:1162–70. doi:10.1038/ni.3026
85. Liu SQ, Jiang S, Li C, Zhang B, Li QJ. miR-17-92 cluster targets phosphatase and tensin homology and Ikaros family zinc finger 4 to promote TH17-mediated inflammation. *J Biol Chem* (2014) 289:12446–56. doi:10.1074/jbc.M114.550723
86. Singh PB, Pua HH, Happ HC, Schneider C, von Moltke J, Locksley RM, et al. MicroRNA regulation of type 2 innate lymphoid cell homeostasis

- and function in allergic inflammation. *J Exp Med* (2017) 214:3627–43. doi:10.1084/jem.20170545
87. Yu D, Rao S, Tsai LM, Lee SK, He Y, Sutcliffe EL, et al. The transcriptional repressor Bcl-6 directs T follicular helper cell lineage commitment. *Immunity* (2009) 31:457–68. doi:10.1016/j.immuni.2009.07.002
 88. Han YC, Vidigal JA, Mu P, Yao E, Singh I, Gonzalez AJ, et al. An allelic series of miR-17 approximately 92-mutant mice uncovers functional specialization and cooperation among members of a microRNA polycistron. *Nat Genet* (2015) 47:766–75. doi:10.1038/ng.3321
 89. Serr I, Furst RW, Ott VB, Scherm MG, Nikolaev A, Gokmen F, et al. miRNA92a targets KLF2 and the phosphatase PTEN signaling to promote human T follicular helper precursors in T1D islet autoimmunity. *Proc Natl Acad Sci U S A* (2016) 113:E6659–68. doi:10.1073/pnas.1606646113
 90. Banerjee A, Schambach F, DeJong CS, Hammond SM, Reiner SL. MicroRNA-155 inhibits IFN-gamma signaling in CD4+ T cells. *Eur J Immunol* (2010) 40:225–31. doi:10.1002/eji.200939381
 91. Haasch D, Chen YW, Reilly RM, Chiou XG, Koterski S, Smith ML, et al. T cell activation induces a noncoding RNA transcript sensitive to inhibition by immunosuppressant drugs and encoded by the proto-oncogene, BIC. *Cell Immunol* (2002) 217:78–86. doi:10.1016/S0008-8749(02)00506-3
 92. Rodriguez A, Vigorito E, Clare S, Warren MV, Couttet P, Soond DR, et al. Requirement of bic/microRNA-155 for normal immune function. *Science* (2007) 316:608–11. doi:10.1126/science.1139253
 93. Thai TH, Calado DP, Casola S, Ansel KM, Xiao C, Xue Y, et al. Regulation of the germinal center response by microRNA-155. *Science* (2007) 316:604–8. doi:10.1126/science.1141229
 94. Escobar TM, Kanellopoulou C, Kugler DG, Kilaru G, Nguyen CK, Nagarajan V, et al. miR-155 activates cytokine gene expression in Th17 cells by regulating the DNA-binding protein Jarid2 to relieve polycomb-mediated repression. *Immunity* (2014) 40:865–79. doi:10.1016/j.immuni.2014.03.014
 95. Hu R, Huffaker TB, Kagele DA, Runtsch MC, Bake E, Chaudhuri AA, et al. MicroRNA-155 confers encephalogenic potential to Th17 cells by promoting effector gene expression. *J Immunol* (2013) 190:5972–80. doi:10.4049/jimmunol.1300351
 96. O'Connell RM, Kahn D, Gibson WS, Round JL, Scholz RL, Chaudhuri AA, et al. MicroRNA-155 promotes autoimmune inflammation by enhancing inflammatory T cell development. *Immunity* (2010) 33:607–19. doi:10.1016/j.immuni.2010.09.009
 97. Kohlhaas S, Garden OA, Scudamore C, Turner M, Okkenhaug K, Vigorito E. Cutting edge: the Foxp3 target miR-155 contributes to the development of regulatory T cells. *J Immunol* (2009) 182:2578–82. doi:10.4049/jimmunol.0803162
 98. Lu LF, Thai TH, Calado DP, Chaudhry A, Kubo M, Tanaka K, et al. Foxp3-dependent microRNA155 confers competitive fitness to regulatory T cells by targeting SOCS1 protein. *Immunity* (2009) 30:80–91. doi:10.1016/j.immuni.2008.11.010
 99. Kuchen S, Resch W, Yamane A, Kuo N, Li Z, Chakraborty T, et al. Regulation of microRNA expression and abundance during lymphopoiesis. *Immunity* (2010) 32:828–39. doi:10.1016/j.immuni.2010.05.009
 100. Huffaker TB, Hu R, Runtsch MC, Bake E, Chen X, Zhao J, et al. Epistasis between microRNAs 155 and 146a during T cell-mediated antitumor immunity. *Cell Rep* (2012) 2:1697–709. doi:10.1016/j.celrep.2012.10.025
 101. Rusca N, Deho L, Montagner S, Zielinski CE, Sica A, Sallusto F, et al. miR-146a and NF-kappaB1 regulate mast cell survival and T lymphocyte differentiation. *Mol Cell Biol* (2012) 32:4432–44. doi:10.1128/MCB.00824-12
 102. Yang L, Boldin MP, Yu Y, Liu CS, Ea CK, Ramakrishnan P, et al. miR-146a controls the resolution of T cell responses in mice. *J Exp Med* (2012) 209:1655–70. doi:10.1084/jem.20112218
 103. Li B, Wang X, Choi IY, Wang YC, Liu S, Pham AT, et al. miR-146a modulates autoreactive Th17 cell differentiation and regulates organ-specific autoimmunity. *J Clin Invest* (2017a) 127(10):3702–16. doi:10.1172/JCI94012
 104. Lu LF, Boldin MP, Chaudhry A, Lin LL, Taganov KD, Hanada T, et al. Function of miR-146a in controlling Treg cell-mediated regulation of Th1 responses. *Cell* (2010) 142:914–29. doi:10.1016/j.cell.2010.08.012
 105. Mohnle P, Schutz SV, van der Heide V, Hubner M, Luchting B, Sedlbauer J, et al. MicroRNA-146a controls Th1-cell differentiation of human CD4+ T lymphocytes by targeting PRKCEpsilon. *Eur J Immunol* (2015) 45:260–72. doi:10.1002/eji.201444667
 106. Zhou Q, Haupt S, Kreuzer JT, Hammitzsch A, Proft F, Neumann C, et al. Decreased expression of miR-146a and miR-155 contributes to an abnormal Treg phenotype in patients with rheumatoid arthritis. *Ann Rheum Dis* (2015) 74:1265–74. doi:10.1136/annrheumdis-2013-204377
 107. Zhao JL, Rao DS, Boldin MP, Taganov KD, O'Connell RM, Baltimore D. NF-kappaB dysregulation in microRNA-146a-deficient mice drives the development of myeloid malignancies. *Proc Natl Acad Sci U S A* (2011) 108:9184–9. doi:10.1073/pnas.1105398108
 108. Hu R, Kagele DA, Huffaker TB, Runtsch MC, Alexander M, Liu J, et al. miR-155 promotes T follicular helper cell accumulation during chronic, low-grade inflammation. *Immunity* (2014) 41:605–19. doi:10.1016/j.immuni.2014.09.015
 109. Liu WH, Kang SG, Huang Z, Wu CJ, Jin HY, Maine CJ, et al. A miR-155-Peli1-c-Rel pathway controls the generation and function of T follicular helper cells. *J Exp Med* (2016) 213(9):1901–19. doi:10.1084/jem.20160204
 110. Crepeau RL, Zhang P, Usherwood EJ. MicroRNA miR-155 is necessary for efficient gammaherpesvirus reactivation from latency, but not for establishment of latency. *J Virol* (2016) 90:7811–21. doi:10.1128/JVI.00521-16
 111. Baumjohann D, Preite S, Reboldi A, Ronchi F, Ansel KM, Lanzavecchia A, et al. Persistent antigen and germinal center B cells sustain T follicular helper cell responses and phenotype. *Immunity* (2013b) 38:596–605. doi:10.1016/j.immuni.2012.11.020
 112. Deenick EK, Chan A, Ma CS, Gatto D, Schwartzberg PL, Brink R, et al. Follicular helper T cell differentiation requires continuous antigen presentation that is independent of unique B cell signaling. *Immunity* (2010) 33:241–53. doi:10.1016/j.immuni.2010.07.015
 113. Linterman MA, Vinuesa CG. Signals that influence T follicular helper cell differentiation and function. *Semin Immunopathol* (2010) 32:183–96. doi:10.1007/s00281-009-0194-z
 114. Rolf J, Bell SE, Kovacs D, Janas ML, Soond DR, Webb LM, et al. Phosphoinositide 3-kinase activity in T cells regulates the magnitude of the germinal center reaction. *J Immunol* (2010) 185(7):4042–52. doi:10.4049/jimmunol.1001730
 115. Jeker LT, Zhou X, Gershberg K, de Kouchkovsky D, Morar MM, Stadthagen G, et al. MicroRNA 10a marks regulatory T cells. *PLoS One* (2012) 7:e36684. doi:10.1371/journal.pone.0036684
 116. Takahashi H, Kanno T, Nakayama S, Hirahara K, Sciume G, Muljo SA, et al. TGF-beta and retinoic acid induce the microRNA miR-10a, which targets Bcl-6 and constrains the plasticity of helper T cells. *Nat Immunol* (2012) 13:587–95. doi:10.1038/ni.2286
 117. Ripamonti A, Provasi E, Lorenzo M, De Simone M, Ranzani V, Vangelisti S, et al. Repression of miR-31 by BCL6 stabilizes the helper function of human follicular helper T cells. *Proc Natl Acad Sci U S A* (2017) 114:12797–802. doi:10.1073/pnas.1705364114
 118. Li HB, Tong J, Zhu S, Batista PJ, Duffy EE, Zhao J, et al. m(6)A mRNA methylation controls T cell homeostasis by targeting the IL-7/STAT5/SOCS pathways. *Nature* (2017b) 548:338–42. doi:10.1038/nature23450
 119. Sandberg R, Neilson JR, Sarma A, Sharp PA, Burge CB. Proliferating cells express mRNAs with shortened 3' untranslated regions and fewer microRNA target sites. *Science* (2008) 320:1643–7. doi:10.1126/science.1155390
 120. Whisenant TC, Peralta ER, Aarberg LD, Gao NJ, Head SR, Ordoukhanian P, et al. The activation-induced assembly of an RNA/protein interactome centered on the splicing factor U2AF2 regulates gene expression in human CD4 T cells. *PLoS One* (2015) 10:e0144409. doi:10.1371/journal.pone.0144409

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2018 Baumjohann and Heissmeyer. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



Follicular Helper T Cells in Systemic Lupus Erythematosus

Sun Jung Kim, Kyungwoo Lee and Betty Diamond*

The Feinstein Institute for Medical Research, Northwell Health, New York, NY, United States

CD4⁺ follicular helper T (T_{fh}) cells constitute a subset of effector T cells that participate in the generation of high-affinity humoral responses. They express the chemokine receptor CXCR5 and produce the cytokine IL-21, both of which are required for their contribution to germinal center formation. Uncontrolled expansion of T_{fh} cells is observed in various mouse models of systemic autoimmune diseases and in patients with these diseases. In particular, the frequency of circulating T_{fh} is correlated with disease activity and anti-DNA antibody titer in patients with systemic lupus erythematosus. Recent studies reveal functional diversity within the T_{fh} population in both humans and mice. We will summarize here the molecular mechanisms for T_{fh} cell generation, survival and function in both humans and mice, and the relationship between T_{fh} cells and autoimmune disease in animal models and in patients.

Keywords: follicular helper T cells, autoimmunity, transcription factors, human immunology, lupus models

OPEN ACCESS

Edited by:

Georgia Foustier,
San Raffaele Hospital
(IRCCS), Italy

Reviewed by:

Koji Yasutomo,
Tokushima University, Japan
Lewis Zhichang Shi,
Case Western Reserve University,
United States

*Correspondence:

Betty Diamond
bdiamond@northwell.edu

Specialty section:

This article was submitted
to T Cell Biology,
a section of the journal
Frontiers in Immunology

Received: 17 May 2018

Accepted: 20 July 2018

Published: 03 August 2018

Citation:

Kim SJ, Lee K and Diamond B (2018)
Follicular Helper T Cells in Systemic
Lupus Erythematosus.
Front. Immunol. 9:1793.
doi: 10.3389/fimmu.2018.01793

INTRODUCTION

It has long been known that T cells are required for successful humoral immune responses (1). Upon stimulation, CD4⁺ naïve T cells differentiate into T helper (Th) 1, Th2, Th17, follicular helper T (T_{fh}), and regulatory T (T_{reg}) cells. Each subset requires distinct activation signals and cytokine milieu during activation; each expresses a unique transcriptional profile with a unique master regulator and distinct effector cytokines; and each subset serves a different function in the immune response. Activation of naïve CD4⁺ T cells by antigen-presenting cells (APCs) together with IL-6 and IL-21 induces T_{fh} cells. Development of T_{fh} cells is required for an optimal antibody response, mainly through activation and maintenance of the germinal center (GC) response. T_{fh} cells express the master transcription factor, B cell lymphoma 6 (BCL6). This transcription factor distinguishes the T_{fh} compartment from other T helper cell subsets, and is required for the maintenance of their effector function in lymphoid organs (2–7). Activation of STAT3 is also important for T_{fh} differentiation as it upregulates BCL6 (8). STAT3-deficient mice have a diminished T_{fh} compartment and an increased Th1 response. The expression of BCL6 is antagonized by the transcriptional repressor, B lymphocyte-induced maturation protein 1 (BLIMP1) (4). Induction of BCL6 and the downregulation of BLIMP1 appear to be equally important in human T_{fh} differentiation (4, 8).

Follicular helper T cells express a unique set of effector molecules that are critical for their function. High expression of CXCR5 with downregulation of CCR7 has been shown to be important for migration of T_{fh} into the B cell follicle (9). Inducible co-stimulator (ICOS), programmed cell death protein 1 (PD-1), and CD40L are also expressed on T_{fh} cells and these molecules are required for activation of B cells (6, 10–14). Mice lacking CD40 or CD40L exhibit disrupted GC responses and impaired long-term memory (12). ICOS–ICOSL signaling is essential for a sustained T:B interaction; consequently, loss of either molecule impairs GC B cell survival and plasma cell differentiation (15). PD-1 deficiency does not affect the number of GC B cells and the early antibody response; however, engagement of PD-1 on T_{fh} cells and PD-L1/2 on GC B cells is critical for the development of long-lived plasma cells (13). T_{fh} cells produce high amounts of

IL-21 and IL-4 which are required for proliferation of B cells and immunoglobulin class switching (6, 14). Blockade or deficiency of these effector molecules abrogates the generation of an effective humoral response.

INDUCTION PROGRAM OF Tfh CELLS

The immediate precursor of Tfh cells is not fully described. Initial studies identified the cytokines, IL-21 and IL-6, as key molecules in triggering Tfh cell differentiation. Engagement of TCR on naïve CD4⁺ T cells with peptide/MHC II on APCs together with IL-21 induces expression of CXCR5 and a low level of BCL6 expression *in vivo* (16, 17). Both IL-6 and IL-21 can induce *Bcl6* mRNA *in vitro* (5); both activate cells through the STAT3 pathway (8, 16). IL-6 and IL-21 can trigger the early Tfh differentiation program in CD4⁺ T cells, but an absolute requirement for IL-6 and IL-21 has been challenged. IL-6^{-/-}, IL-21^{-/-}, or IL-21R^{-/-} mice develop Tfh cells normally following immunization with protein antigen or viral infection (18, 19). Although the triggering signals for first induction of BCL6 and CXCR5 in Tfh cells are not fully understood, once T cells acquire a “CXCR5^{lo}BCL6^{lo}” Tfh signature (pre-Tfh), some will migrate to the T cell–B cell border (9, 20). A second transcription factor, c-MAF, is induced concomitantly with BCL6 (21). C-MAF has also shown to induce CXCR5. BCL6 and c-MAF cooperatively induce ICOS, PD-1, and CXCR4, suggesting both molecules orchestrate a core transcriptional program in Tfh cells (22).

CXCR5^{lo}BCL6^{lo} pre-Tfh cells interact with cognate B cells at the T–B zone to induce a high level of BCL6 and CXCR5. This allows stable localization of the cells in follicles and sustains mature Tfh cell differentiation (4, 23). Signaling from a homodimeric interaction of signaling lymphocytic activation molecule (SLAM)-associated protein (SAP) (SH2D1A) on B cells leads to induction of the SLAM family receptor, CD84, promoting stable T:B interactions (23, 24). In the absence of SAP, pre-Tfh cells develop normally, but fail to move into the GC and mature to GC-Tfh cells (24). This B cell-dependent Tfh differentiation can be bypassed by chronic immune activation. Mice lacking MHC II expression on B cells develop normal GC-Tfh cells following repeated immunization (25) or chronic viral infection (26). These observations suggest that while B cells maybe the major APC important in Tfh differentiation, B-independent Tfh maturation can occur when a high and sustained amount of antigen is present. An ICOS–ICOSL interaction between pre-Tfh and B cells is required for maintaining a high level of CXCR5 or BCL6 in Tfh cells. ICOS signaling activates the PI3K pathway and selective abrogation of ICOS–PI3K signaling dramatically reduces Tfh differentiation (27). ICOS–PI3K signaling keeps pre-Tfh cell motile at the T cell–B cell border to facilitate cognate T:B interactions (28). It also augments IL-4 and IL-21 transcription (27, 29). The importance of the PI3K pathway during Tfh differentiation is demonstrated in studies of mice with CD4-specific deletion of a microRNA miR 17-92. miR17-92 is induced at an early stage of Tfh cell differentiation and regulates PI3K signaling intensity through downregulation of phosphatase, PHLPP2. T cells with a deletion of miR17-92 show a severe reduction in Tfh differentiation (30).

NEGATIVE REGULATION OF Tfh BY FOLLICULAR REGULATORY T (Tfr) CELLS

The interaction between Tfh cells and B cells (GC B cells and plasma cells) needs to be precisely regulated to ensure proper immune activation and to limit excessive inflammation and autoimmunity. Tfr cells, a recently identified Treg subset, migrate to the GC and inhibit Tfh cells and GC B cells (31, 32). Differentiation of Tfr is mediated by recognition of antigens presented on DCs in lymphoid organs (31). Signals from the co-stimulatory molecules CD28 and ICOS are essential for Tfr differentiation as *Cd28*^{-/-} and *Icos*^{-/-} mice lack Tfr cells (32, 33). Engagement of CTLA-4 on Tfr cells with B7.1 and B7.2 on APCs is critical for their suppressive mechanism (34–36). In contrast, Tfr cells express high levels of PD-1 which mitigates the suppressive function of Tfr cells. *Pdcd1*^{-/-} Tfr cells suppress antibody production more potently *in vitro* and *in vivo* (33). Tfr cells express CXCR5 which guides them to the GC (32). Tfr, like Tfh cells, also express the canonical transcription factor, BCL6, although the level of BCL6 is lower than in Tfh cells. In addition to BCL6, Tfr cells express FOXP3 and BLIMP1, which are not expressed in Tfh cells (37).

The Tfh:Tfr ratio controls antibody responses. In the basal-state, Tfr cells constitute approximately 50% of all CD4⁺CXCR5⁺ T cells, resulting in a 1:1 ratio of Tfh:Tfr cells. Under stimulatory conditions including immunization or infection, Tfh cells expand resulting in a lower proportion of Tfr cells. A proper differentiation of Tfr is critical for immune tolerance as mice with Tfr deficiency (*Bcl6*^{fl/ΔFoxp3}-CRE) develop spontaneous autoimmune disease (38). A critical role for Tfr but not other Treg cells in antibody production was confirmed in an adoptive transfer study. Transfer of Tregs from Tfr-deficient *Bcl6*^{-/-} or wild-type mice together with CD4⁺ T cells into *Tcrb*^{-/-} mice resulted in an expansion of Tfh cells and higher antibody responses (37).

Tfh IN B CELL ACTIVATION AND IN GC AND IN THE EXTRAFOLLICULAR SPACE

The primary function of Tfh cells is to help activation and differentiation of antigen-specific B cells in a protective immune response. This requires engagement of surface molecules (CD40L and ICOS) on Tfh cells with their ligands on B cells (CD40 and ICOSL, respectively) and secretion of cytokines (IL-21 and IL-4) from Tfh cells (6, 14). GCs are the primary site of B cell affinity maturation and class switching. Tfh cells regulate GC size, restrict low affinity B cell entry into the GC, and support and select high-affinity B cells during affinity maturation within the GC (4, 13, 29, 39). The quality and quantity of help provided by Tfh cells regulates B cell clonal expansion. As restriction of T cell help to high-affinity B cells is required for affinity maturation in GCs (40), proper regulation of Tfh determines the outcome of the GC response.

Signals from Tfh cell to B cells are required for both the generation and the maintenance of GCs. To initiate GC B cell differentiation, Tfh cells induce expression of BCL6 (master transcription factor for GC B cells) in activated B cells. The precise mechanism how the initial BCL6 expression occurs is complex and not clearly understood; however, signals from the IL-21R are

an important factor for BCL6 expression in B cells (18, 41). Tfh cells also provide proliferation and survival signals to GC B cells *via* multiple pathways, including CD40L, PD-1, IL-21, and IL-4 (41–44). The CD40–CD40L interaction is important in survival of GC B cells partly because it also helps to induce BCL6 (45). Combinatorial signals by CD40L and IL-21 or CD40L and IL-4 maintain GC B cell proliferation. Although PD-1 is known to provide a potent inhibitory signal to T cells (46), deficiency in PD-1 or PD-L1/2 reduces B cell differentiation (13). Formation of GCs is normal in the absence of PD-1 or PD-L1/2, but maintenance of GCs is severely affected due to an increase in apoptosis of GC B cells. The interaction between ICOS on Tfh cells and ICOSL on B cells is important for both B cells and Tfh cells (10, 11). The ICOS–ICOSL signaling is essential for the sustained T:B interaction, consequently influencing affinity maturation of GC B cells, survival, and plasma cell differentiation (15). Cognate interaction between Tfh and GC B cells is a key mechanism for selection of high-affinity GC B cells and for memory B cells or plasma cells (47, 48). Tfh cells regulate plasmablast emergence out of GC during the early stages of GC reaction. IL-21 produced from Tfh cells and TNFSF13 (APRIL) produced from podoplanin⁺ CD157^{hi} fibroblastic reticular cells are the two main factors for this process (49). IL-21 is highly expressed by Tfh cells and is the most potent cytokine for driving plasma cell differentiation (2, 50). Both IL-21 and IL-4 are class switch factors for IgG1 (51, 52).

Follicular helper T cells play a key role in B cell differentiation into antibody-producing cells outside GCs as well. Recent studies demonstrate that there are different subsets of Tfh cells in humans and mice. BCL6⁺ Tfh cells are required for B cell priming for extrafollicular antibody production in a T-dependent immune response (53). In contrast to the conventional Tfh cells, they express PD-1 but not CXCR5 and appear before GC formation at the T cell–B cell border. IL-21 produced from these PD-1⁺ Tfh cells support B cell activation and differentiation to antibody-producing cells.

REPERTOIRE OF Tfh

How the repertoire of Tfh cells is determined is not well understood, and an altered repertoire of Tfh cells can contribute to the development of autoimmune diseases. Our recent study demonstrated that the repertoire of Tfh is different in lupus-prone mice compared to healthy control mice, and the alteration of Tfh repertoire is closely associated with lupus development (54). This study suggests that not only the number of Tfh cells, but also the antigenic specificity of Tfh cells is likely essential to immune tolerance.

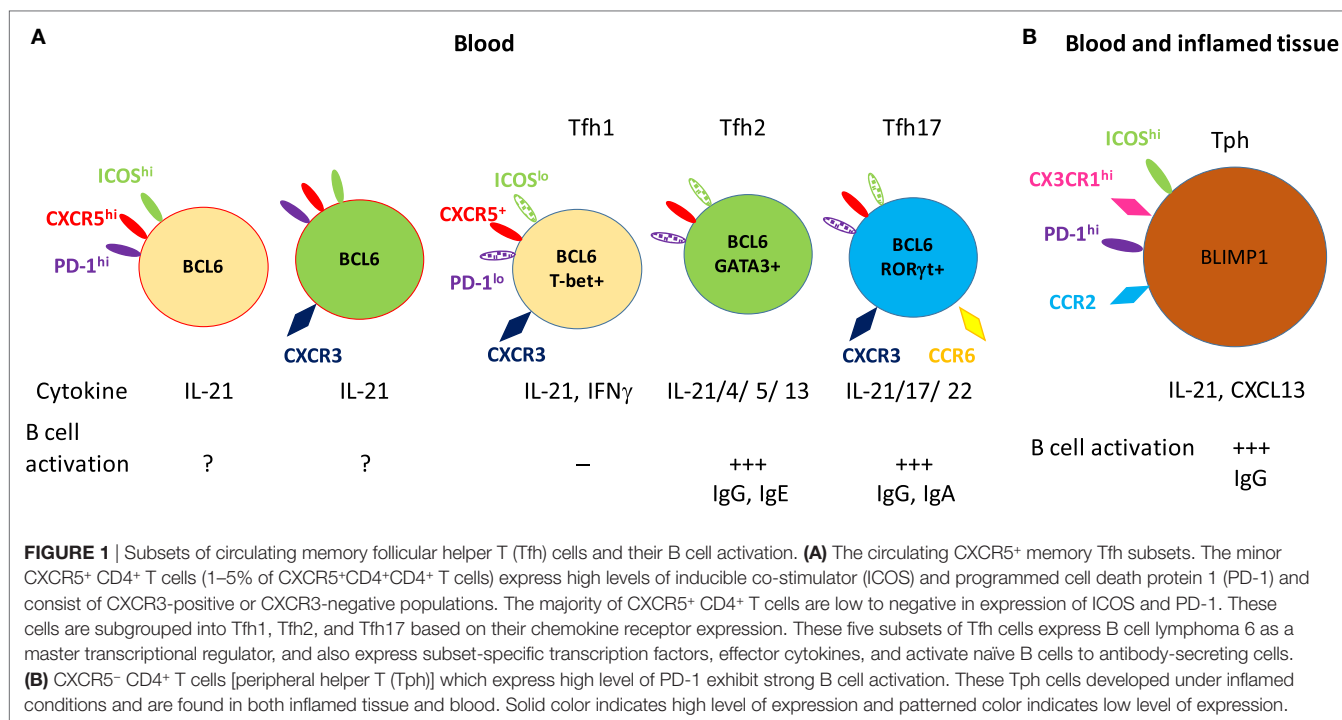
Sets of antigenic peptides presented by APCs, including thymic epithelial cells, dendritic cells (DCs), and B cells are likely to influence the repertoire of T cells emerging from the thymus. In the periphery, affinity and duration of interaction between TCR and peptide/MHC II influences DCs to induce the activation of T cells and modulate the repertoire of T cells (25, 55). Cathepsin S (CTSS) is a major endoprotease cleaving the invariant chain from MHC II molecules and also cleaving exogenous antigens (54). Increased activity of CTSS in DCs is involved in regulation of the Tfh repertoire (54). In one study, the antigenic specificity of Tfh cells was investigated during a polyclonal B cell response

in mice (56). Polyreactive Tfh cells are generated and individual antigen-specific Tfh cells show distinct cytokine profiles. A study of human Tfh shows that CXCR5⁺ circulating memory-like Tfh cells reactive with influenza protein preferentially recognize peptide epitopes from hemagglutinin, while CXCR5[−] non-Tfh preferentially recognize nucleoprotein (57). This study suggests that different effector T cell subsets may activate distinct B cells. Together, these observations, while limited, support an important role for the TCR repertoire of Tfh cells in autoreactive B cell selection and/or activation.

HUMAN Tfh

Human Tfh cells are characterized in tonsil by high expression of CXCR5 and ICOS (58). These CXCR5^{hi}ICOS^{hi} cells reside in follicles and induce B cells to become antibody-secreting plasma cells, which is a hallmark of *bona fide* Tfh cells. In healthy individuals, tonsillar CD4⁺ T cells contain distinct populations according to the expression of CXCR5 and ICOS, including CXCR5^{hi}ICOS^{hi} (GC-Tfh), CXCR5^{lo}ICOS^{hi}, CXCR5^{lo}ICOS^{lo}, and CXCR5[−]ICOS[−]. These subpopulations of Tfh cells display differential activation of B cells. CXCR5^{hi}ICOS^{hi} Tfh cells effectively activate and induce antibody production of GC-B cells (IgD[−]CD38⁺CD19⁺) and memory B cells (IgD[−]CD38[−]CD19⁺) but not naïve B cells (IgD⁺CD38[−]CD19⁺) (58, 59). CXCR5^{lo}ICOS^{lo} Tfh cells show robust proliferation and differentiation of naïve B cells and memory B cells but not GC B cells (60). Neither CXCR5[−]ICOS[−] Tfh nor CXCR5^{lo}ICOS^{hi} Tfh cells show B cell activation activity. Each subpopulation of Tfh cells also produces a distinct pattern of cytokines. Upon co-culture with B cells or stimulated by anti-CD3/28, CXCR5^{hi}ICOS^{hi} GC-Tfh cells secrete high level of IL-21 and CXCL13 but low level of IL-10 and IL-4, CXCR5^{lo}ICOS^{lo} Tfh cells secrete high levels of IL-21 and IL-10 with low level of IL-4, and CXCR5^{lo}ICOS^{hi} Tfh cells secrete high level of IL-17A instead of IL-21 and IL-10. These studies suggest each Tfh population possesses a unique B cell activation capacity.

B cell lymphoma 6-expressing extrafollicular Tfh cells have been identified in tissues and in blood (60, 61). Tfh cells identified in blood are different from tonsillar Tfh cells phenotypically and functionally. Circulating Tfh cells do not have same patterns of surface markers (CXCR5, ICOS, and PD-1) as tonsillar Tfh cells. Recent studies suggest that blood CXCR5⁺ CD4⁺ T cells represent a memory compartment of Tfh lineage cells. Extensive analysis of these cells revealed functionally and phenotypically distinct subsets, identified by expression of ICOS, PD-1, CCR7, CXCR3, and CCR6. While the expression of BCL6 and ICOS is high in tonsillar GC-Tfh cells, both ICOS and BCL6 protein expression is low in blood Tfh cells (61, 62). Less than 1% of blood CXCR5⁺ Tfh cells are ICOS⁺ and PD-1^{hi} in healthy individuals (63, 64). The majority of CXCR5⁺ Tfh cells does not express ICOS and can be subsetted based on CXCR3, CCR6, and CCR7 expression, and, as mentioned above, each subgroup activates distinctive B cell subsets and expresses different gene expression profiles (**Figure 1**). The CXCR3⁺ CCR6[−] subset (Tfh1) expresses the transcription factor T-bet and produces IFN γ ; the CXCR3[−] CCR6[−] subset (Tfh2) expresses GATA3 and produces IL-4, IL-5, and IL-13; and the CXCR3[−] CCR6⁺ subset (Tfh17) expresses ROR γ t and produces IL-17A and IL-22 (61). All subsets of Tfh cells produce



IL-21 but not all induce B cells to secrete immunoglobulin; each subset has a distinct capacity for B cell activation. Naïve B cells can be stimulated by Tfh2 and Tfh17 but not CXCR3⁺ Tfh1 cells (61). Tfh2 cells promote IgG and IgE, while Tfh17 cells efficient inducers of IgG and IgA production. These studies suggest different Tfh subsets regulate Ig class switching these subsets appear to exist in humans but not in mice.

The mechanism of human Tfh cell differentiation is actively being investigated. Human Tfh cells are differentiated from naïve CD4⁺ T cells, however, the critical signals and effector molecules are not fully understood. The plasticity among CD4⁺ helper T cell subsets is also poorly understood. Recent *in vitro* studies identified some molecules which influence human Tfh differentiation. Initial studies demonstrated that DCs can induce differentiation of Tfh cells from naïve CD4⁺ T cells from human peripheral blood mononuclear cell (PBMCs) or from cord blood. Among the cytokines which are produced from activated DCs, IL-12 is the most efficient cytokine to induce Tfh-related molecules (CXCR5, ICOS, and IL-21) and Tfh-related transcription factors (BCL6, BATF, and cMAF) (65). Another study found IL-12 most efficiently induced Tfh cells to produce IL-21 to activate B cells to become antibody-secreting cells (66). Surprisingly, these studies found that the Tfh-inducing cytokines in mouse, IL-6 or IL-21, are less efficient in the human system. IL-12 mediated human Tfh cells contain classical Th1 phenotypes, displaying a mixed population of IL-21⁺IFN γ [−] Tfh and IL-21⁺IFN γ ⁺ Tfh cells. The importance of IL-12 in Tfh cell differentiation is also supported by the studies of patients with impaired IL-12 signaling. Patients with deficiency in IL-12R β 1, TYK2, STAT3, but not STAT1 exhibit compromised IL-12-induced expression of IL-21 by CD4⁺ T cells (67). Although IL-12/STAT3 axis is critical for IL-21 and BCL6 expression, it is a dispensable for ICOS expression.

Defects in generating Tfh cells from STAT3 mutant CD4⁺ T cells could contribute to the impaired T-dependent humoral immune responses observed in patients with STAT3 mutations (68).

Another cytokine, TGF β , was shown to have a unique function in human Tfh cell differentiation (69). TGF β and IL-12 together specifically down-regulate the level of BLIMP1 in Tfh cells. This regulatory mechanism is not shared by mouse Tfh cells. TGF β also induces CXCL13 expression in human naïve CD4⁺ T cells (70), and, therefore, may contribute to the accumulation of T and B cell aggregates (ectopic GCs) in inflammatory tissues (71).

Activin A is an inducible molecule that is broadly expressed in immune cells. Signals *via* CD40 and toll-like receptors induce DCs to upregulate Activin A expression (72); Activin A induces human naïve CD4⁺ T cells to produce CXCL13 and differentiate into Tfh cells *in vitro* (73). Therefore, Activin A may be also involved in Tfh differentiation especially under inflammatory conditions. A recent study on systemic lupus erythematosus (SLE) suggests the involvement of OX40L⁺ monocytes and DCs in Tfh production. The frequency of OX40L⁺ monocytes correlated with disease activity and the frequency of ICOS⁺ PD-1⁺ Tfh cells in blood (74). TNFSF4, the gene encoding OX40L, has been determined by GWAS to have risk alleles in SLE, rheumatoid arthritis (RA), and multiple sclerosis (75), further suggesting an involvement of OX40 and OX40L interaction mediated Tfh pathway in disease conditions. The molecular mechanisms by which naïve human CD4⁺ T cells differentiate to Tfh cells or maintain Tfh characteristics remain largely unknown.

RELEVANCE TO AUTOIMMUNITY

Helper T cells are required for a protective immune response and for the development of autoantibodies (76). Development

of autoantibodies is abrogated by genetic deletion of MHC II in CD4⁺ T cells in the B6. *lpr* lupus mouse model (77). Helper T cell activity, especially Tfh cell activity, directly correlates with GC formation (78–80). Abnormalities in Tfh cells (Tfh cell accumulation or functional alteration of Tfh cells) have been observed in various models of autoimmune diseases. In *sanroque* mice, the number of Tfh cells is increased with the hyperactive ICOS signaling and excessive production of IL-21, leading to the development of spontaneous GC formation and lupus-like autoimmune phenotypes (3, 81, 82). Roquin has been shown to post-transcriptionally regulate the expression of ICOS and OX40, which are highly expressed by Tfh cells (58, 81, 83). CD4⁺ T cells lacking Roquin overexpress ICOS and OX40, promoting Tfh cell differentiation. Blocking ICOS/ICOS-L or CD40/CD40L interactions ameliorates disease progression in autoimmune mouse models (84, 85). High serum levels of IL-21 were also detected in BXSb-Yaa mice (2), and blockade of the IL-21/IL-21R pathway slows the progression of lupus by decreasing lymphocyte activation and circulating IgG1 levels in BXSb-Yaa mice (86). These data demonstrate IL-21 as a key molecule for B cell differentiation to plasma cells. In this model, both follicular and extrafollicular T cells are important producers of IL-21. Another SLE model, MRL/*lpr* mice, has increased extrafollicular ICOS^{hi}PSGL1^{lo} CD4⁺ T cells in secondary lymphoid organs. These cells are the primary source of CD40L and IL-21 supporting extrafollicular IgG plasmablasts (10). CD40L expressed by Tfh cells contributes to activation of B cells in autoimmune animal models. Antigen-specific extra Tfh cells which share phenotypic characteristics with Tfh also mediate IgG secretion through IL-21 in a CD40L-dependent manner (10). Autoreactive Tfh cells have been observed in K/BxN mice, too. In this mice, self-reactive CD4⁺ T cells escape clonal deletion in the thymus and appear in periphery. The primed self-reactive CD4⁺ T cells help B cells to produce pathologic antibodies (87). Tfh cells have been shown to contribute to the pathogenesis of lupus through ICOS–B7RP-1 pathway in NZB/NZW F1 mice (88). Abnormalities in Tfh cells are also observed in mice with genetic manipulations in non-T cells. Mice with BLIMP1-deficient DCs spontaneously develop lupus-like phenotypes in a gender-specific manner, and these mice have increased Tfh cells and GC B cells (89). This study also shows that an increased proinflammatory cytokine, IL-6 from the BLIMP1-deficient DCs facilitates Tfh differentiation as haploinsufficiency for IL-6, prevents lupus-related phenotypes including Tfh cells.

There are multiple lines of evidences showing that aberrant Tfh-cell and GC responses are associated with human SLE. The majority of IgG+ autoantibodies in patients are somatically mutated, an observation consistent with an involvement of GCs, the site of action of Tfh (90, 91). In lupus nephritis lesions, Tfh-like cells expressing ICOS, PD-1, BCL-6, and IL-21 were observed, forming ectopic GCs (92). Furthermore, an increased population of circulating CXCR5⁺ICOS⁺PD-1⁺ Tfh cells was identified in a subset of SLE patients; this increase correlated with disease activity but not necessarily with the titer of anti-DNA antibodies (93, 94). ICOS⁺PD-1⁺ Tfh cells secrete high levels of IL-21, and a strong correlation between the expression of ICOS or PD-1 and plasmablast number has been observed. The differential

expression of ICOS, PD-1, and CCR7 further defines subpopulations within the subsets (recently activated: ICOS⁺PD-1⁺CCR7^{lo} and quiescent: ICOS⁺PD-1⁺CCR7^{hi}) (62, 63). Other studies employing a combinatorial analysis of chemokine receptors (CXCR3 and CCR6) have defined three major subsets (Tfh1, Tfh2, and Tfh17) (61, 69). These analyses identified a relative dominance of Tfh2 and/or Tfh17 subsets over Tfh1 in systemic diseases, including SLE (93, 95), IgG4-related disease (96), and organ-specific diseases such as [Sjogren's syndrome (97), RA (98, 99), and autoimmune thyroid disease (100)], and the neurological diseases [myasthenia gravis (101), multiple sclerosis (102), and neuromyelitis optica (103)]. The alterations in Tfh subsets often positively correlated with disease activity and/or serum autoantibody titer, and with the frequency of circulating plasmablasts. These observations support the association of an expanded Tfh response with the pathogenesis of human autoimmune diseases; however, how the increased Tfh response leads to activation of autoreactive B cells is not elucidated in humans yet.

A recent study identified a new PD-1^{hi} helper T cell subset in peripheral tissue [peripheral helper T (Tph) cells] from patients with RA (104). Tph cells are expanded in inflamed joint and blood from RA patients and display unusual biological features. They are programmed to infiltrate part of the inflamed body (CCR2⁺), and stimulate B cells to produce antibodies (IL-21 and CXCL13) *in situ*. In contrast to CXCR5⁺ Tfh cells, Tph cells exhibit a unique profile of molecules; CXCR5⁺ but BLIMP1^{hi} (high ratio of BLIMP1:BCL6) and PD-1^{hi} (104). This finding expands the spectrum of T cells that are associated with inflammatory diseases; it will be interesting to investigate whether PD-1^{hi} Tph cells are expanded in other autoimmune and inflammatory diseases.

CONCLUSION

Compelling data now demonstrate the key role of the Tfh in B cell responses, both protective and pathogenic. Much still remains to be learned. For example, we do not know whether the subsets we have identified represent activation or differentiation states, and whether there is phenotypic and functional plasticity among any of these subsets. We do not know how Tph relate to Tfh and whether and how they contribute to tissue inflammation. We need to learn whether uncontrolled antibody responses relate to an expanded Tfh number or an altered Tfh repertoire and we need to learn if Tfh selectively respond to particular antigens. The field is rapidly growing and answers to these, and answers to these questions, and more, are likely to be forthcoming and to suggest strategies for treatment of both autoimmune diseases and immunodeficiency states.

AUTHOR CONTRIBUTIONS

BD organizes and supervises the entire manuscript. SK contributes for human section. KL contributes for mouse section.

FUNDING

This study was supported by grant NIH NIAMS R01 AR065209.

REFERENCES

- Dejaco C, Duftner C, Grubeck-Loebenstein B, Schirmer M. Imbalance of regulatory T cells in human autoimmune diseases. *Immunology* (2006) 117(3): 289–300. doi:10.1111/j.1365-2567.2005.02317.x
- Ozaki K, Spolski R, Ettinger R, Kim HP, Wang G, Qi CF, et al. Regulation of B cell differentiation and plasma cell generation by IL-21, a novel inducer of Blimp-1 and Bcl-6. *J Immunol* (2004) 173(9):5361–71. doi:10.4049/jimmunol.173.9.5361
- Linterman MA, Rigby RJ, Wong RK, Yu D, Brink R, Cannons JL, et al. Follicular helper T cells are required for systemic autoimmunity. *J Exp Med* (2009) 206(3):561–76. doi:10.1084/jem.20081886
- Johnston RJ, Poholek AC, DiToro D, Yusuf I, Eto D, Barnett B, et al. Bcl6 and Blimp-1 are reciprocal and antagonistic regulators of T follicular helper cell differentiation. *Science* (2009) 325(5943):1006–10. doi:10.1126/science.1175870
- Nurieva RI, Chung Y, Martinez GJ, Yang XO, Tanaka S, Matskevitch TD, et al. Bcl6 mediates the development of T follicular helper cells. *Science* (2009) 325(5943):1001–5. doi:10.1126/science.1176676
- Yu D, Rao S, Tsai LM, Lee SK, He Y, Sutcliffe EL, et al. The transcriptional repressor Bcl-6 directs T follicular helper cell lineage commitment. *Immunity* (2009) 31(3):457–68. doi:10.1016/j.immuni.2009.07.002
- Liu X, Yan X, Zhong B, Nurieva RI, Wang A, Wang X, et al. Bcl6 expression specifies the T follicular helper cell program in vivo. *J Exp Med* (2012) 209(10):S1–24. doi:10.1084/jem.20120219
- Ray JP, Marshall HD, Laidlaw BJ, Staron MM, Kaech SM, Craft J. Transcription factor STAT3 and type I interferons are corepressive insulators for differentiation of follicular helper and T helper 1 cells. *Immunity* (2014) 40(3): 367–77. doi:10.1016/j.immuni.2014.02.005
- Haynes NM, Allen CD, Lesley R, Ansel KM, Killeen N, Cyster JG. Role of CXCR5 and CCR7 in follicular Th cell positioning and appearance of a programmed cell death gene-1-high germinal center-associated subpopulation. *J Immunol* (2007) 179(8):5099–108. doi:10.4049/jimmunol.179.8.5099
- Odegar JM, Marks BR, DiPlacido LD, Poholek AC, Kono DH, Dong C, et al. ICOS-dependent extrafollicular helper T cells elicit IgG production via IL-21 in systemic autoimmunity. *J Exp Med* (2008) 205(12):2873–86. doi:10.1084/jem.20080840
- Crotty S. T follicular helper cell differentiation, function, and roles in disease. *Immunity* (2014) 41(4):529–42. doi:10.1016/j.immuni.2014.10.004
- Higuchi T, Aiba Y, Nomura T, Matsuda J, Mochida K, Suzuki M, et al. Cutting edge: ectopic expression of CD40 ligand on B cells induces lupus-like autoimmune disease. *J Immunol* (2002) 168(1):9–12. doi:10.4049/jimmunol.168.1.9
- Good-Jacobson KL, Szumilas CG, Chen L, Sharpe AH, Tomayko MM, Shlomchik MJ. PD-1 regulates germinal center B cell survival and the formation and affinity of long-lived plasma cells. *Nat Immunol* (2010) 11(6):535–42. doi:10.1038/ni.1877
- Avery DT, Bryant VL, Ma CS, de Waal Malefyt R, Tangye SG. IL-21-induced isotype switching to IgG and IgA by human naive B cells is differentially regulated by IL-4. *J Immunol* (2008) 181(3):1767–79. doi:10.4049/jimmunol.181.3.1767
- Liu D, Xu H, Shih C, Wan Z, Ma X, Ma W, et al. T-B-cell entanglement and ICOSL-driven feed-forward regulation of germinal center reaction. *Nature* (2015) 517(7533):214–8. doi:10.1038/nature13803
- Nurieva RI, Chung Y, Hwang D, Yang XO, Kang HS, Ma L, et al. Generation of T follicular helper cells is mediated by interleukin-21 but independent of T helper 1, 2, or 17 cell lineages. *Immunity* (2008) 29(1):138–49. doi:10.1016/j.immuni.2008.05.009
- Suto A, Kashiwakuma D, Kagami S, Hirose K, Watanabe N, Yokote K, et al. Development and characterization of IL-21-producing CD4+ T cells. *J Exp Med* (2008) 205(6):1369–79. doi:10.1084/jem.20072057
- Linterman MA, Beaton L, Yu D, Ramiscal RR, Srivastava M, Hogan JJ, et al. IL-21 acts directly on B cells to regulate Bcl-6 expression and germinal center responses. *J Exp Med* (2010) 207(2):353–63. doi:10.1084/jem.20091738
- Poholek AC, Hansen K, Hernandez SG, Eto D, Chande A, Weinstein JS, et al. In vivo regulation of Bcl6 and T follicular helper cell development. *J Immunol* (2010) 185(1):313–26. doi:10.4049/jimmunol.0904023
- Hardtke S, Ohl L, Forster R. Balanced expression of CXCR5 and CCR7 on follicular T helper cells determines their transient positioning to lymph node follicles and is essential for efficient B-cell help. *Blood* (2005) 106(6):1924–31. doi:10.1182/blood-2004-11-4494
- Andris F, Denanglaire S, Anciaux M, Hercor M, Hussein H, Leo O. The transcription factor c-Maf promotes the differentiation of follicular helper T cells. *Front Immunol* (2017) 8:480. doi:10.3389/fimmu.2017.00480
- Kroenke MA, Eto D, Locci M, Cho M, Davidson T, Haddad EK, et al. Bcl6 and Maf cooperate to instruct human follicular helper CD4 T cell differentiation. *J Immunol* (2012) 188(8):3734–44. doi:10.4049/jimmunol.1103246
- Yusuf I, Kageyama R, Monticelli L, Johnston RJ, Ditoro D, Hansen K, et al. Germinal center T follicular helper cell IL-4 production is dependent on signaling lymphocytic activation molecule receptor (CD150). *J Immunol* (2010) 185(1):190–202. doi:10.4049/jimmunol.0903505
- Qi H, Cannons JL, Klauschen F, Schwartzberg PL, Germain RN. SAP-controlled T-B cell interactions underlie germinal center formation. *Nature* (2008) 455(7214):764–9. doi:10.1038/nature07345
- Deenick EK, Chan A, Ma CS, Gatto D, Schwartzberg PL, Brink R, et al. Follicular helper T cell differentiation requires continuous antigen presentation that is independent of unique B cell signaling. *Immunity* (2010) 33(2):241–53. doi:10.1016/j.immuni.2010.07.015
- Fahey LM, Wilson EB, Elsaesser H, Fistonich CD, McGavern DB, Brooks DG. Viral persistence redirects CD4 T cell differentiation toward T follicular helper cells. *J Exp Med* (2011) 208(5):987–99. doi:10.1084/jem.20101773
- Gigoux M, Shang J, Pak Y, Xu M, Choe J, Mak TW, et al. Inducible costimulator promotes helper T-cell differentiation through phosphoinositide 3-kinase. *Proc Natl Acad Sci U S A* (2009) 106(48):20371–6. doi:10.1073/pnas.0911573106
- Xu H, Li X, Liu D, Li J, Zhang X, Chen X, et al. Follicular T-helper cell recruitment governed by bystander B cells and ICOS-driven motility. *Nature* (2013) 496(7446):523–7. doi:10.1038/nature12058
- Rolf J, Bell SE, Kovacs D, Janas ML, Soond DR, Webb LM, et al. Phosphoinositide 3-kinase activity in T cells regulates the magnitude of the germinal center reaction. *J Immunol* (2010) 185(7):4042–52. doi:10.4049/jimmunol.1001730
- Kang SG, Liu WH, Lu P, Jin HY, Lim HW, Shepherd J, et al. MicroRNAs of the miR-17 approximately 92 family are critical regulators of T(FH) differentiation. *Nat Immunol* (2013) 14(8):849–57. doi:10.1038/ni.2648
- Wollenberg I, Agua-Doce A, Hernandez A, Almeida C, Oliveira VG, Faro J, et al. Regulation of the germinal center reaction by Foxp3+ follicular regulatory T cells. *J Immunol* (2011) 187(9):4553–60. doi:10.4049/jimmunol.1101328
- Linterman MA, Pierson W, Lee SK, Kallies A, Kawamoto S, Rayner TF, et al. Foxp3+ follicular regulatory T cells control the germinal center response. *Nat Med* (2011) 17(8):975–82. doi:10.1038/nm.2425
- Sage PT, Francisco LM, Carman CV, Sharpe AH. The receptor PD-1 controls follicular regulatory T cells in the lymph nodes and blood. *Nat Immunol* (2013) 14(2):152–61. doi:10.1038/ni.2496
- Sage PT, Paterson AM, Lovitch SB, Sharpe AH. The coinhibitory receptor CTLA-4 controls B cell responses by modulating T follicular helper, T follicular regulatory, and T regulatory cells. *Immunity* (2014) 41(6):1026–39. doi:10.1016/j.immuni.2014.12.005
- Wing JB, Ise W, Kurosaki T, Sakaguchi S. Regulatory T cells control antigen-specific expansion of Tfh cell number and humoral immune responses via the coreceptor CTLA-4. *Immunity* (2014) 41(6):1013–25. doi:10.1016/j.immuni.2014.12.006
- Wang CJ, Heuts F, Ovcinnikovs V, Wardzinski L, Bowers C, Schmidt EM, et al. CTLA-4 controls follicular helper T-cell differentiation by regulating the strength of CD28 engagement. *Proc Natl Acad Sci U S A* (2015) 112(2): 524–9. doi:10.1073/pnas.1414576112
- Chung Y, Tanaka S, Chu F, Nurieva RI, Martinez GJ, Rawal S, et al. Follicular regulatory T cells expressing Foxp3 and Bcl-6 suppress germinal center reactions. *Nat Med* (2011) 17(8):983–8. doi:10.1038/nm.2426
- Fu W, Liu X, Lin X, Feng H, Sun L, Li S, et al. Deficiency in T follicular regulatory cells promotes autoimmunity. *J Exp Med* (2018) 215(3):815–25. doi:10.1084/jem.20170901
- Schwicker TA, Vitoria GD, Fooksman DR, Kamphorst AO, Mugnier MR, Gitlin AD, et al. A dynamic T cell-limited checkpoint regulates affinity-dependent B cell entry into the germinal center. *J Exp Med* (2011) 208(6): 1243–52. doi:10.1084/jem.20102477

40. Victora GD, Schwickert TA, Fooksman DR, Kamphorst AO, Meyer-Hermann M, Dustin ML, et al. Germinal center dynamics revealed by multiphoton microscopy with a photoactivatable fluorescent reporter. *Cell* (2010) 143(4):592–605. doi:10.1016/j.cell.2010.10.032
41. Zotos D, Coquet JM, Zhang Y, Light A, D'Costa K, Kallies A, et al. IL-21 regulates germinal center B cell differentiation and proliferation through a B cell-intrinsic mechanism. *J Exp Med* (2010) 207(2):365–78. doi:10.1084/jem.20091777
42. Good KL, Bryant VL, Tangye SG. Kinetics of human B cell behavior and amplification of proliferative responses following stimulation with IL-21. *J Immunol* (2006) 177(8):5236–47. doi:10.4049/jimmunol.177.8.5236
43. Choe J, Kim HS, Zhang X, Armitage RJ, Choi YS. Cellular and molecular factors that regulate the differentiation and apoptosis of germinal center B cells. Anti-Ig down-regulates Fas expression of CD40 ligand-stimulated germinal center B cells and inhibits Fas-mediated apoptosis. *J Immunol* (1996) 157(3):1006–16.
44. Banchereau J, de Paoli P, Valle A, Garcia E, Rousset F. Long-term human B cell lines dependent on interleukin-4 and antibody to CD40. *Science* (1991) 251(4989):70–2. doi:10.1126/science.1702555
45. Randall TD, Heath AW, Santos-Argumedo L, Howard MC, Weissman IL, Lund FE. Arrest of B lymphocyte terminal differentiation by CD40 signaling: mechanism for lack of antibody-secreting cells in germinal centers. *Immunity* (1998) 8(6):733–42. doi:10.1016/S1074-7613(00)80578-6
46. Barber DL, Wherry EJ, Masopust D, Zhu B, Allison JP, Sharpe AH, et al. Restoring function in exhausted CD8 T cells during chronic viral infection. *Nature* (2006) 439(7077):682–7. doi:10.1038/nature04444
47. Wang Y, Shi J, Yan J, Xiao Z, Hou X, Lu P, et al. Germinal-center development of memory B cells driven by IL-9 from follicular helper T cells. *Nat Immunol* (2017) 18(8):921–30. doi:10.1038/ni.3788
48. Weisel FJ, Zuccarino-Catania GV, Chikina M, Shlomchik MJ. A temporal switch in the germinal center determines differential output of memory B and plasma cells. *Immunity* (2016) 44(1):116–30. doi:10.1016/j.immuni.2015.12.004
49. Zhang Y, Tech L, George LA, Acs A, Durrett RE, Hess H, et al. Plasma cell output from germinal centers is regulated by signals from Tfh and stromal cells. *J Exp Med* (2018) 215(4):1227–43. doi:10.1084/jem.20160832
50. Ettinger R, Sims GP, Fairhurst AM, Robbins R, da Silva YS, Spolski R, et al. IL-21 induces differentiation of human naive and memory B cells into antibody-secreting plasma cells. *J Immunol* (2005) 175(12):7867–79. doi:10.4049/jimmunol.175.12.7867
51. Reinhardt RL, Liang HE, Locksley RM. Cytokine-secreting follicular T cells shape the antibody repertoire. *Nat Immunol* (2009) 10(4):385–93. doi:10.1038/ni.1715
52. Ozaki K, Spolski R, Feng CG, Qi CF, Cheng J, Sher A, et al. A critical role for IL-21 in regulating immunoglobulin production. *Science* (2002) 298(5598):1630–4. doi:10.1126/science.1077002
53. Lee SK, Rigby RJ, Zotos D, Tsai LM, Kawamoto S, Marshall JL, et al. B cell priming for extrafollicular antibody responses requires Bcl-6 expression by T cells. *J Exp Med* (2011) 208(7):1377–88. doi:10.1084/jem.20102065
54. Kim SJ, Schatzle S, Ahmed SS, Haap W, Jang SH, Gregersen PK, et al. Increased cathepsin S in Prdm1(-/-) dendritic cells alters the TFH cell repertoire and contributes to lupus. *Nat Immunol* (2017) 18(9):1016–24. doi:10.1038/ni.3793
55. Benson RA, MacLeod MK, Hale BG, Patakas A, Garside P, Brewer JM. Antigen presentation kinetics control T cell/dendritic cell interactions and follicular helper T cell generation in vivo. *Elife* (2015) 4:1–16. doi:10.7554/eLife.06994
56. Leddon SA, Sant AJ. The peptide specificity of the endogenous T follicular helper cell repertoire generated after protein immunization. *PLoS One* (2012) 7(10):e46952. doi:10.1371/journal.pone.0046952
57. Leddon SA, Richards KA, Treanor JJ, Sant AJ. Abundance and specificity of influenza reactive circulating memory follicular helper and non-follicular helper CD4 T cells in healthy adults. *Immunology* (2015) 146(1):157–62. doi:10.1111/imm.12491
58. Rasheed AU, Rahn HP, Sallusto F, Lipp M, Muller G. Follicular B helper T cell activity is confined to CXCR5(hi)ICOS(hi) CD4 T cells and is independent of CD57 expression. *Eur J Immunol* (2006) 36(7):1892–903. doi:10.1002/eji.200636136
59. Bryant VL, Ma CS, Avery DT, Li Y, Good KL, Corcoran LM, et al. Cytokine-mediated regulation of human B cell differentiation into Ig-secreting cells: predominant role of IL-21 produced by CXCR5+ T follicular helper cells. *J Immunol* (2007) 179(12):8180–90. doi:10.4049/jimmunol.179.12.8180
60. Bentebibel SE, Schmitt N, Banchereau J, Ueno H. Human tonsil B-cell lymphoma 6 (BCL6)-expressing CD4+ T-cell subset specialized for B-cell help outside germinal centers. *Proc Natl Acad Sci U S A* (2011) 108(33):E488–97. doi:10.1073/pnas.1100898108
61. Morita R, Schmitt N, Bentebibel SE, Ranganathan R, Bourdery L, Zurawski G, et al. Human blood CXCR5(+)CD4(+) T cells are counterparts of T follicular cells and contain specific subsets that differentially support antibody secretion. *Immunity* (2011) 34(1):108–21. doi:10.1016/j.immuni.2010.12.012
62. He J, Tsai LM, Leong YA, Hu X, Ma CS, Chevalier N, et al. Circulating precursor CCR7(lo)PD-1(hi) CXCR5(+) CD4(+) T cells indicate Tfh cell activity and promote antibody responses upon antigen reexposure. *Immunity* (2013) 39(4):770–81. doi:10.1016/j.immuni.2013.09.007
63. Locci M, Havenar-Daughton C, Landais E, Wu J, Kroenke MA, Arlehamn CL, et al. Human circulating PD-1+CXCR3-CXCR5+ memory Tfh cells are highly functional and correlate with broadly neutralizing HIV antibody responses. *Immunity* (2013) 39(4):758–69. doi:10.1016/j.immuni.2013.08.031
64. Bentebibel SE, Lopez S, Obermoser G, Schmitt N, Mueller C, Harrod C, et al. Induction of ICOS+CXCR3+CXCR5+ TH cells correlates with antibody responses to influenza vaccination. *Sci Transl Med* (2013) 5(176):176ra32. doi:10.1126/scitranslmed.3005191
65. Schmitt N, Morita R, Bourdery L, Bentebibel SE, Zurawski SM, Banchereau J, et al. Human dendritic cells induce the differentiation of interleukin-21-producing T follicular helper-like cells through interleukin-12. *Immunity* (2009) 31(1):158–69. doi:10.1016/j.immuni.2009.04.016
66. Ma CS, Suryani S, Avery DT, Chan A, Nanan R, Santner-Nanan B, et al. Early commitment of naive human CD4(+) T cells to the T follicular helper (TFH) cell lineage is induced by IL-12. *Immunol Cell Biol* (2009) 87(8):590–600. doi:10.1038/icb.2009.64
67. Ma CS, Avery DT, Chan A, Batten M, Bustamante J, Boisson-Dupuis S, et al. Functional STAT3 deficiency compromises the generation of human T follicular helper cells. *Blood* (2012) 119(17):3997–4008. doi:10.1182/blood-2011-11-392985
68. McIlwain DR, Grusdat M, Pozdeev VI, Xu HC, Shinde P, Reardon C, et al. T-cell STAT3 is required for the maintenance of humoral immunity to LCMV. *Eur J Immunol* (2015) 45(2):418–27. doi:10.1002/eji.201445060
69. Schmitt N, Liu Y, Bentebibel SE, Munagala I, Bourdery L, Venuprasad K, et al. The cytokine TGF-beta co-opts signaling via STAT3-STAT4 to promote the differentiation of human TFH cells. *Nat Immunol* (2014) 15(9):856–65. doi:10.1038/ni.2947
70. Kobayashi S, Watanabe T, Suzuki R, Furu M, Ito H, Ito J, et al. TGF-beta induces the differentiation of human CXCL13-producing CD4(+) T cells. *Eur J Immunol* (2016) 46(2):360–71. doi:10.1002/eji.201546043
71. Bugatti S, Manzo A, Vitolo B, Benaglio F, Binda E, Scarabelli M, et al. High expression levels of the B cell chemoattractant CXCL13 in rheumatoid synovium are a marker of severe disease. *Rheumatology (Oxford)* (2014) 53(10):1886–95. doi:10.1093/rheumatology/keu163
72. Robson NC, Phillips DJ, McAlpine T, Shin A, Svobodova S, Toy T, et al. Activin-A: a novel dendritic cell-derived cytokine that potently attenuates CD40 ligand-specific cytokine and chemokine production. *Blood* (2008) 111(5):2733–43. doi:10.1182/blood-2007-03-080994
73. Locci M, Wu JE, Arumemi F, Mikulski Z, Dahlberg C, Miller AT, et al. Activin A programs the differentiation of human TFH cells. *Nat Immunol* (2016) 17(8):976–84. doi:10.1038/ni.3494
74. Jacquemin C, Schmitt N, Contin-Bordes C, Liu Y, Narayanan P, Seneschal J, et al. OX40 ligand contributes to human lupus pathogenesis by promoting T follicular helper response. *Immunity* (2015) 42(6):1159–70. doi:10.1016/j.immuni.2015.05.012
75. Stranger BE, De Jager PL. Coordinating GWAS results with gene expression in a systems immunologic paradigm in autoimmunity. *Curr Opin Immunol* (2012) 24(5):544–51. doi:10.1016/j.coi.2012.09.002
76. Sobel ES, Cohen PL, Eisenberg RA. Ipr T cells are necessary for autoantibody production in lpr mice. *J Immunol* (1993) 150(9):4160–7.

77. Sobel ES, Kakkanaiah VN, Kakkanaiah M, Cheek RL, Cohen PL, Eisenberg RA. T-B collaboration for autoantibody production in lpr mice is cognate and MHC-restricted. *J Immunol* (1994) 152(12):6011–6.
78. William J, Euler C, Christensen S, Shlomchik MJ. Evolution of autoantibody responses via somatic hypermutation outside of germinal centers. *Science* (2002) 297(5589):2066–70. doi:10.1126/science.1073924
79. Fazilleau N, McHeyzer-Williams LJ, Rosen H, McHeyzer-Williams MG. The function of follicular helper T cells is regulated by the strength of T cell antigen receptor binding. *Nat Immunol* (2009) 10(4):375–84. doi:10.1038/ni.1704
80. Nutt SL, Tarlinton DM. Germinal center B and follicular helper T cells: siblings, cousins or just good friends? *Nat Immunol* (2011) 12(6):472–7. doi:10.1038/ni.2019
81. Vinuesa CG, Cook MC, Angelucci C, Athanasopoulos V, Rui L, Hill KM, et al. A RING-type ubiquitin ligase family member required to repress follicular helper T cells and autoimmunity. *Nature* (2005) 435(7041):452–8. doi:10.1038/nature03555
82. Yu D, Tan AH, Hu X, Athanasopoulos V, Simpson N, Silva DG, et al. Roquin represses autoimmunity by limiting inducible T-cell co-stimulator messenger RNA. *Nature* (2007) 450(7167):299–303. doi:10.1038/nature06253
83. Vogel KU, Edelmann SL, Jeltsch KM, Bertossi A, Heger K, Heinz GA, et al. Roquin paralogs 1 and 2 redundantly repress the Icos and Ox40 costimulator mRNAs and control follicular helper T cell differentiation. *Immunity* (2013) 38(4):655–68. doi:10.1016/j.immuni.2012.12.004
84. Iwai H, Abe M, Hirose S, Tsushima F, Tezuka K, Akiba H, et al. Involvement of inducible costimulator-B7 homologous protein costimulatory pathway in murine lupus nephritis. *J Immunol* (2003) 171(6):2848–54. doi:10.4049/jimmunol.171.6.2848
85. Kalled SL, Cutler AH, Datta SK, Thomas DW. Anti-CD40 ligand antibody treatment of SNF1 mice with established nephritis: preservation of kidney function. *J Immunol* (1998) 160(5):2158–65.
86. Bubier JA, Sproule TJ, Foreman O, Spolski R, Shaffer DJ, Morse HC III, et al. A critical role for IL-21 receptor signaling in the pathogenesis of systemic lupus erythematosus in BXSB-Yaa mice. *Proc Natl Acad Sci U S A* (2009) 106(5):1518–23. doi:10.1073/pnas.0807309106
87. Perera J, Liu X, Zhou Y, Joseph NE, Meng L, Turner JR, et al. Insufficient autoantigen presentation and failure of tolerance in a mouse model of rheumatoid arthritis. *Arthritis Rheum* (2013) 65(11):2847–56. doi:10.1002/art.38085
88. Hu YL, Metz DP, Chung J, Siu G, Zhang M. B7RP-1 blockade ameliorates autoimmunity through regulation of follicular helper T cells. *J Immunol* (2009) 182(3):1421–8. doi:10.4049/jimmunol.182.3.1421
89. Kim SJ, Zou YR, Goldstein J, Reizis B, Diamond B. Tolerogenic function of Blimp-1 in dendritic cells. *J Exp Med* (2011) 208(11):2193–9. doi:10.1084/jem.20110658
90. Arce E, Jackson DG, Gill MA, Bennett LB, Banachereau J, Pascual V. Increased frequency of pre-germinal center B cells and plasma cell precursors in the blood of children with systemic lupus erythematosus. *J Immunol* (2001) 167(4):2361–9. doi:10.4049/jimmunol.167.4.2361
91. Jacobi AM, Odendahl M, Reiter K, Bruns A, Burmester GR, Radbruch A, et al. Correlation between circulating CD27high plasma cells and disease activity in patients with systemic lupus erythematosus. *Arthritis Rheum* (2003) 48(5):1332–42. doi:10.1002/art.10949
92. Liarski VM, Kaverina N, Chang A, Brandt D, Yanez D, Talasnik L, et al. Cell distance mapping identifies functional T follicular helper cells in inflamed human renal tissue. *Sci Transl Med* (2014) 6(230):230ra46. doi:10.1126/scitranslmed.3008146
93. Choi JY, Ho JH, Pasoto SG, Bunin V, Kim ST, Carrasco S, et al. Circulating follicular helper-like T cells in systemic lupus erythematosus: association with disease activity. *Arthritis Rheumatol* (2015) 67(4):988–99. doi:10.1002/art.39020
94. Zhang X, Lindwall E, Gauthier C, Lyman J, Spencer N, Alarakhia A, et al. Circulating CXCR5+CD4+helper T cells in systemic lupus erythematosus patients share phenotypic properties with germinal center follicular helper T cells and promote antibody production. *Lupus* (2015) 24(9):909–17. doi:10.1177/0961203314567750
95. Simpson N, Gatenby PA, Wilson A, Malik S, Fulcher DA, Tangye SG, et al. Expansion of circulating T cells resembling follicular helper T cells is a fixed phenotype that identifies a subset of severe systemic lupus erythematosus. *Arthritis Rheum* (2010) 62(1):234–44. doi:10.1002/art.25032
96. Akiyama M, Suzuki K, Yamaoka K, Yasuoka H, Takeshita M, Kaneko Y, et al. Number of circulating follicular helper 2 T cells correlates with IgG4 and interleukin-4 levels and plasmablast numbers in IgG4-related disease. *Arthritis Rheumatol* (2015) 67(9):2476–81. doi:10.1002/art.39209
97. Li XY, Wu ZB, Ding J, Zheng ZH, Li XY, Chen LN, et al. Role of the frequency of blood CD4(+) CXCR5(+) CCR6(+) T cells in autoimmunity in patients with Sjogren's syndrome. *Biochem Biophys Res Commun* (2012) 422(2):238–44. doi:10.1016/j.bbrc.2012.04.133
98. Wang J, Shan Y, Jiang Z, Feng J, Li C, Ma L, et al. High frequencies of activated B cells and T follicular helper cells are correlated with disease activity in patients with new-onset rheumatoid arthritis. *Clin Exp Immunol* (2013) 174(2):212–20. doi:10.1111/cei.12162
99. Arroyo-Villa I, Bautista-Caro MB, Balsa A, Aguado-Acin P, Bonilla-Hernan MG, Plasencia C, et al. Constitutively altered frequencies of circulating follicular helper T cell counterparts and their subsets in rheumatoid arthritis. *Arthritis Res Ther* (2014) 16(6):500. doi:10.1186/s13075-014-0500-6
100. Zhu C, Ma J, Liu Y, Tong J, Tian J, Chen J, et al. Increased frequency of follicular helper T cells in patients with autoimmune thyroid disease. *J Clin Endocrinol Metab* (2012) 97(3):943–50. doi:10.1210/jc.2011-2003
101. Luo C, Li Y, Liu W, Feng H, Wang H, Huang X, et al. Expansion of circulating counterparts of follicular helper T cells in patients with myasthenia gravis. *J Neuroimmunol* (2013) 256(1–2):55–61. doi:10.1016/j.jneuroim.2012.12.001
102. Zhang X, Liu S, Chang T, Xu J, Zhang C, Tian F, et al. Intrathymic Th/B cells interaction leads to ectopic GCs formation and anti-AChR antibody production: central role in triggering MG occurrence. *Mol Neurobiol* (2016) 53(1):120–31. doi:10.1007/s12035-014-8985-1
103. Fan X, Jiang Y, Han J, Liu J, Wei Y, Jiang X, et al. Circulating memory T follicular helper cells in patients with neuromyelitis optica/neuromyelitis optica spectrum disorders. *Mediators Inflamm* (2016) 2016:3678152. doi:10.1155/2016/3678152
104. Rao DA, Gurish MF, Marshall JL, Slowikowski K, Fonseka CY, Liu Y, et al. Pathologically expanded peripheral T helper cell subset drives B cells in rheumatoid arthritis. *Nature* (2017) 542(7639):110–4. doi:10.1038/nature20810

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2018 Kim, Lee and Diamond. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



Several Follicular Regulatory T Cell Subsets With Distinct Phenotype and Function Emerge During Germinal Center Reactions

Nicolas Fazilleau^{1,2,3,4} and Meryem Aloulou^{1,2,3,4*}

¹ Centre de Physiopathologie de Toulouse Purpan, Toulouse, France, ² INSERM U1043, Toulouse, France, ³ CNRS UMR5282, Toulouse, France, ⁴ Université Toulouse III Paul-Sabatier, Toulouse, France

An efficient B cell immunity requires a dynamic equilibrium between positive and negative signals. In germinal centers (GCs), T follicular helper cells are supposed to be the positive regulator while T follicular regulatory (Tfr) cells were assigned to be the negative regulators. Indeed, Tfr cells are considered as a homogenous cell population dedicated to dampen the GC extent. Moreover, Tfr cells prevent autoimmunity since their dysregulation leads to production of self-reactive antibodies (Ab). However, a growing corpus of evidence has revealed additional and unexpected functions for Tfr cells in the regulation of B cell responses. This review provides an overview of the Tfr cell contribution and presents Tfr cell proprieties in the context of vaccination.

Keywords: T follicular regulatory cells, germinal centers, heterogeneity, subsets, T follicular helper cells

OPEN ACCESS

Edited by:

Georgia Fousteri,
San Raffaele Hospital
(IRCCS), Italy

Reviewed by:

Alex Dent,
Purdue University Indianapolis,
United States
Luis Graca,
Universidade de Lisboa, Portugal

*Correspondence:

Meryem Aloulou
meryem.aloulou@inserm.fr

Specialty section:

This article was submitted
to T Cell Biology,
a section of the journal
Frontiers in Immunology

Received: 05 June 2018

Accepted: 20 July 2018

Published: 13 August 2018

Citation:

Fazilleau N and Aloulou M (2018)
Several Follicular Regulatory
T Cell Subsets With Distinct
Phenotype and Function Emerge
During Germinal Center Reactions.
Front. Immunol. 9:1792.
doi: 10.3389/fimmu.2018.01792

INTRODUCTION

One of the key roles of humoral response is to clear pathogens and to prevent future pathogen assaults through the induction of immune memory. This long-term protection is largely mediated by the generation of high-affinity and neutralizing antibodies (Ab) bearing the suitable isotype for pathogen clearance. However, the increase of B cell receptors affinity in germinal centers (GCs) is mediated by somatic hypermutation, which is a random process mediated by the enzyme activation-induced cytidine deaminase. Therefore, affinity maturation requires tight regulation processes of mutagenesis and B cell selection that are essential in GCs to guarantee proper surveillance and to avoid sustained activation that could lead to autoimmunity or inflammatory diseases. GCs represent critical sites within secondary lymphoid organ (SLO) in which B cell responses are amplified and refined in specificity and isotype, leading to the generation of high-affinity memory B cells and long-lived plasma cells (PCs). The cellular mechanisms that control positive selection of GC B cells have been mostly elucidated. Entanglement of B cells with T follicular helper (Tfh) cells is at the center of this selection process (1). Indeed, Tfh cells seed primordial GCs and provide positive help to the selected GC B cells bearing high-affinity Ab. Several signals provided by Tfh cells lead to B cell maturation (2). Tfh cells bear T cell receptor (TCR) with high affinity for the immunizing antigen (Ag), which lead to stable interaction with GC B cells bearing abundant complexes of foreign peptide-MHC complexes (pMHCII) (3–5). T-B cell entanglement rarely lasts more than 10–15 min but triggers the activation of signaling cascades in B cells and cytokine secretion by Tfh cells that promote survival, proliferation, mutagenesis, and terminal differentiation of B cells into PCs and memory B cells (1, 2). This interaction also provides additional helper signals from Tfh cells through co-stimulatory molecules (2). Furthermore,

TABLE 1 | Impact of Tfr cell abnormalities on B cell maturation.

Tfr cell abnormality	Ag model	Impact on B cell response	Reference
Tfr deficiency (<i>Bcl6</i> ^{-/-} Treg and WT naïve Th cells transferred into <i>Tcr</i> ^{-/-} mice)	NP-KLH in CFA	Increase high-affinity IgG1, IgG2a, and IgG2b Ab	(6)
Tfr deficiency (bone marrow chimeras <i>SAP</i> ^{-/-} : <i>Foxp3</i> ^{DTT})	NP-KLH in SAS	Reduce high-affinity Ab after boost	(7)
Tfr deficiency (<i>CXCR5</i> ^{-/-} Treg and OVA specific Th cells transferred into <i>Tcr</i> ^{-/-} mice)	OVA in Alum	Increase Ag-specific IgM and IgG2b Ab	(8)
Treg cells-lacked IL-10 production (<i>Foxp3cre</i> × <i>IL-10</i> ^{fl/fl})	LCMV Armstrong infection	Reduce LCMV-specific Abs bearing IgG1 and IgG2a isotype	(10)
PD-1 deficiency (WT Tfh and <i>Pdcd1</i> ^{-/-} Tfr cells transfer into <i>CD28</i> ^{-/-})	NP-OVA in CFA	Reduction of Ag-specific Ab	(61)
CTLA-4 deficiency (<i>Foxp3cre</i> ERT2 × <i>Ctla-4</i> ^{fl/fl} Tfh and Tfr cells transfer into <i>CD28</i> ^{-/-} and tamoxifen administration)	NP-OVA in CFA	Increase Ag-specific IgG	(76)
Tfr deficiency (<i>Bcl6</i> ^{-/-} <i>CD25</i> ⁺ Th cells and WT naïve Th cells transferred into <i>CD3</i> ^{-/-} mice)		Reduce IgA titers Reduce IgA maturation and microbiota diversification	(83)
Tfr deficiency (<i>Foxp3cre</i> × <i>Bcl6</i> ^{fl/fl})	– HIV vaccine model – NP-KLH in Alum	Reduce Ab avidity Reduce Ag-specific IgG Increase Ag-specific IgA	(85)

SAS, Sigma Adjuvant System; NP-KLH, 4-hydroxy-3-nitrophenyl-Keyhole Limpet Hemocyanin; OVA, Ovalbumin, CFA, Complement Freund's Adjuvant; SAP, Signaling lymphocytic activation molecules (SLAM)-associated protein; LCMV, Lymphocytic Choriomeningitis; Tfr, T follicular regulatory; CTLA-4, cytotoxic T-lymphocyte antigen 4; *Bcl6*, B-cell lymphoma 6; PD-1, protein cell death 1; Treg, regulatory T cells; Tfh, T follicular helper.

the output of GCs varies depending on the nature of the Ag and the type of inflammation. Several immunoglobulin isotypes exist, which classes are directed by the type of pathogen and the inflammatory context. In addition, specialized subsets of *Foxp3*⁺ regulatory T cells (Treg), the T follicular regulatory (Tfr) T cells, were also recently found in GCs of mice (6–8) and human (9), where they play an immunosuppressive function. Tfr cells express cytotoxic T-lymphocyte antigen 4 (CTLA-4), glucocorticoid-induced tumor necrosis factor receptor (GITR), inducible T-cell co-stimulator (ICOS) and produce IL-10, a phenotype that is the characteristic of activated Treg. Until now, the regulation processes of B cell selection have only been assigned to Tfh cells. However, a recent study has shown the ability of Tfr cells to promote proliferation of GC B cells through IL-10 provision (10). In addition, conflicting results were obtained regarding the role of Tfr cells in controlling affinity maturation of B cells in response to a foreign Ag (see Table 1) (6, 7). All these observations highlight the multifaceted role of Tfr cells and the requirement for further studies to unravel their exact functional proprieties.

Tfh CELLS, THE POSITIVE REGULATORS OF GCs

After initial priming with Ag-experienced dendritic cells (DC), Ag-specific Th cells are clonally selected, expand drastically and, depending on the cytokine milieu and co-stimulatory signals, develop into different lineages of effector T cells such as Th1, Th2, and Th17 or into a lineage of suppressor cells, the periphery Tregs (pTreg). While it has long been thought that Th2 cells were the specific helper of B cells, it is now clear that the guidance of B cell responses is under the control of specific cognate regulators, namely the Tfh cells (2, 11, 12). Early analyses revealed a specific transcription profile for Tfh cells, which was distinct from those of Th1-, Th2-, and Th17-polarized cells and identified a suite of key surface markers that discriminate Tfh cells from the other

effector Th cell lineages (13). Several studies have shown that the repressor B-cell lymphoma 6 (*Bcl6*) drives the genetic program imprinted in Tfh cells (14–17). *Bcl6* expression is dependent on different transcription factors that regulate the key targets of human and mouse Tfh cell formation such as achaete-scute homolog 2 (18), signal transducer and activator of transcription 3 (Stat3) (19), IFN regulatory factor 4 (20, 21), and c-Maf (22). Phenotypically mouse Tfh cells are *CCR7*^{lo} *CXCR5*^{hi} (23, 24) and *CD45RA*⁻ *CXCR5*⁺ cells are also greatly enriched in human tonsils and located in the B follicle areas of these inflamed tissues (25). In addition to these surface molecules that define their strategic anatomical position, Tfh cells highly express molecules essential for their B cell helper function. These molecules include protein cell death 1 (PD-1), B and T lymphocyte attenuator, CD40L, ICOS, SAP, and the production of IL-21 and IL-4 (2). ICOS engagement is important for IL-21 production by Tfh cells (26). However, additional cytokines such as IFN- γ , IL-13, IL-5, and IL-17 can be produced by the polarized Tfh cells depending on the inflammatory context (27). These cytokines promote B cell isotype switch to different pathogen challenges (28). Interestingly, under conditions of intense polarization mouse GC Tfh cells can express Th1-, Th2-, or Th17-differentiation program such as T-bet (T-box expressed in T cells) and IFN- γ , IL-5 and IL-13 or Ror γ t (retinoic acid receptor-related orphan nuclear receptor gamma t) and IL-17, respectively (29–32). In human, few GC Tfh cells display polarized phenotype according to the production of non-Tfh cell cytokines in SLO (33, 34). However, circulating Tfh cells express chemokine receptors corresponding to the polarized non-Tfh cell subsets Th1, Th2, and Th17 cells (26, 27). Indeed, circulating human Tfh cell compartment can be stratified into three distinct polarized subsets based on their expression of chemokine receptors: *CXCR3*⁺*CCR6*⁻ Tfh1-, *CXCR3*⁻*CCR6*⁻ Tfh2-, and *CXCR3*⁻*CCR6*⁺ Tfh17-like cells (26, 27). Overall, depending on the inflammatory context, different Tfh cell profiles are observed reflecting the heterogeneity of the Tfh cell compartment either in mice or human.

Tfr CELLS, OTHER GC REGULATORS

Tfr Cell Development and Antigen-Specificity

Regulatory T cells form the main population of immunosuppressive T cells that plays a pivotal role in maintaining immune self-tolerance and homeostasis by suppressing aberrant or excessive immune responses deleterious to the host (35). Treg use the appropriate homing receptors to control their migration to the site of inflammation and use relevant immunosuppressive mechanisms. In order to repress Th1-, Th2-, and Th17-mediated immune responses, Treg have been shown to co-opt selective aspects of the differentiation programs required for these Th cell lineages (36–38). In the context of B cell responses, Treg were also shown to have the capacity to express Bcl-6 and the chemokine receptor CXCR5, which allows migrating into CXCL13 rich areas to control the GC response (39, 40). These cells were coined as Tfr cells. Using Treg depletion and adoptive transfer, it was initially proposed that Tfr cells derive only from thymus-derived Treg (tTreg) (6–8). However, we recently demonstrated that Tfr cells can also derive from naïve Th cells, a process that required PD-1 ligand 1 (PD-L1) signaling (41). Such differentiation occurs if the adjuvant used is one that supports naïve Th cell conversion to pTreg such as incomplete Freund's adjuvant (IFA) (41). Tfh cells regulate GC B cells by interacting with these cells in a cognate fashion, demonstrating that Tfh cells are specific for the Ag against which the ongoing immune response is mounted. Regarding the Ag-specificity of Tfr cells, our studies also demonstrated that a fraction of these cells could be specific for the immunizing Ag, irrespective of whether it is a self or a foreign Ag (41). Our observations were recently challenged by the fact that it was found that the TCR repertoires of Tfh cells and Tfr cells were shown to be largely distant (42). Moreover, it was also shown the TCR repertoire of Tfr cells was the closest to the one of Treg (42). Finally, only few ovalbumin (OVA)-specific Tfr cells could be detected using pMHCII tetramers in the draining lymph nodes 11 days post-immunization with OVA/IFA (42). Overall, the authors concluded that Tfr cells and Tfh cells do not share the same Ag-specificity and that Tfr cells originate only from tTreg and bear auto-reactive TCRs to suppress autoimmunity (43). We suggest that the inflammatory environment and the nature of the Ag actually both dictate whether Tfr cells can arise from pTreg, which ultimately influence the Tfh and Tfr TCR repertoire overlap and the proportion of Tfr cells sharing the same Ag-specificity with Tfh cells. Indeed, tTreg are thought to be largely auto-reactive (44). Nonetheless, tTreg specific for foreign epitopes have also been described in the naïve population (45), as well as during infection (46, 47). More precisely, the thymic origin of Treg specific for a non-self Ag have been investigated in naïve mice by using tetramer-based enrichment method (45). This corroborates previous studies showing that the TCR repertoire of the Treg population is as diverse as the one of conventional Th cells (48–50), which explains the capacity of tTreg TCRs to possibly cross-react with pMHCII complexes presenting foreign Ag. In the context of *Leishmania major* or *Influenza* infection, Treg were shown to strongly proliferate suggesting that the TCRs of these cells

recognized microbe derive Ag (46, 51, 52). Moreover, in the context of *Mycobacterium tuberculosis* (Mtb) infection (46), it was clearly demonstrated using pMHCII tetramers that Mtb-specific Treg expanded from the pre-existing pool of tTreg and displayed distinct TCR repertoire as compared to the one of the Th cells while they were sharing the same Ag-specificity to Mtb. Elegantly, the authors also showed that a recombinant strain of *Listeria monocytogenes* (Lm) expressing the Mtb immunodominant ESAT6 epitope induced the proliferation of ESAT6-specific conventional Th cells but not of ESAT6-specific Treg, suggesting that the inflammatory milieu of Mtb, but not of Lm, promotes the expansion of Ag-specific tTreg (46). Finally, in the context of self-reactivity, it was shown that myelin oligodendrocyte glycoprotein (46)-specific tTreg expressed TCR of higher avidity than conventional Th cells, suggesting that, despite the same Ag-specificity, their TCR repertoires were different (53). Overall, these data suggest that the control of humoral responses may be defined by distinct Tfr cell subsets, either specific or not for the immunizing Ag, and ultimately GC B cells could be regulated by Tfr cells through non-cognate and cognate interactions.

Tfr Cell Differentiation

The transcriptional program essential for Tfr cells formation was recently described. Most of the genes are common with the Tfh cell program such as Bcl-6, Stat3, and Tcf-1 (54), but specific genes to the Tfr cell lineage are also found such as Nfat2 that initiates CXCR5 expression on Treg (55). Mechanistically, mTOR kinase complexes 1 and 2 (mTOR1 and mTOR2) are involved in Tfh and Tfr cell differentiation. More precisely, both mTOR1 and mTOR2 are essential for Tfh cell formation by linking immune signals to anabolic metabolism and transcriptional activity (56, 57). In addition, mTOR1, but not mTOR2, mediates Tfr cell differentiation by activating the Stat3/Tcf-1/Bcl-6 axis (54). Similar to Tfh cells, initial Tfr cell formation requires engagement of several surface molecules such as CD28, receptors associated to SAP and ICOS that all lead to sustained interaction with Ag-presenting cells (APC) such as DC or B cells. T-cell priming through CD28 is the first signal required for Tfh and Tfr cell development (7, 58), while the adaptor protein SAP enables the formation of stable interaction with B cells essential for Tfh and Tfr cell differentiation (7, 59). ICOS leads to sustained Bcl-6 expression by Tfh and Tfr cells through activation of p85 α regulatory subunit of the PI3-kinase and intracellular osteopontin (60).

In order to prevent full suppression of the GC reaction, a panel of negative regulators was also shown to counterbalance the positive signals that lead to Tfr cell differentiation. PD-1 limits both the differentiation and suppressive function of Tfr cells after their binding to PD-L1 but not to PD-L2 (61). Unlike Tfr cells, PD-1 deficiency has no effect on GC Th cell number, while frequency of circulating Tfr and Tfh cells are greater in the blood, suggesting that both Tfh and Tfr cells are repressed by PD-1 signaling (61). The helix-loop-helix proteins Id2 and Id3 are other suppressive mechanisms of Tfr cell development. Initial TCR engagement of Treg decreases the abundance of Id2 and Id3, which both contribute to the activation of the Tfr cell specific transcription program (62). Interestingly, in contrast to

fully differentiated Tfh cells, Tfr cells co-express the antagonistic regulators B-lymphocyte-induced maturation protein 1 (Blimp1) and Bcl-6. Such co-expression could limit the number of Tfr cell as highlighted by Blimp1 deficiency that does not alter Tfh cell development but causes an increase of the Tfr cell proportion (7). This observation is in contrast with published data showing that Blimp1 directly limits global follicular T cell formation (14, 63), however, its impact on Tfh and Tfr cells separately has not been explored.

Recent studies have described the influence of cytokines on Tfr cell differentiation and maintenance. IL-21/IL21-receptor interaction limits the proliferation of Tfr cells (64). In this study, the authors demonstrated that IL-21 restricts Tfr proliferation by limiting CD25 expression and responsiveness to IL-2, through a Bcl-6-dependent mechanism (64). Furthermore, IL21R-deficiency in mice and human increases Treg and Tfr cell numbers (64). In another series of study, Botta et al. showed that IL-2 prevents Tfr cell development through Blimp1 mechanisms (65) and that Tfr cells express low level of CD25. However, Tfr cells are not completely irresponsiveness to IL-2. Tfr cells express high amount of intermediate affinity IL2-R, CD122 (65), which could promote the IL-2–STAT5 axis important for maintaining Foxp3 expression. Consequently, both Tfh and Tfr cell developments are restrained by IL-2 cytokine. In the context of *Influenza* infection, the presence of high amount of IL-2 in the early phase leads to complete abrogation of Tfr cell formation while Tfh cells are maintained (65). In this context, it was shown that Tfr cells appear laterally once the immune response resolves, which may contribute to prevent the expansion of self-reactive B cells. By contrast, in the context of protein vaccination, the Tfr cells follow the same formation and resolution kinetics than Tfh cells (7, 41), which could result from a different IL-2 profile. Therefore, the dynamic of Tfr cell development does not carry a single form but is closely related to the inflammatory context.

T follicular regulatory cells, like Tfh cells, can exit the draining SLO and join the circulation in both mice (66) and human (67, 68). The circulating Tfr cells (cTfr) have been shown to expand after protein immunization or viral infection. cTfr development requires priming by DC in draining SLO, these cells leave the SLO before GC formation (66). In human, cTfr cells can also be generated before T–B interaction, as they are maintained in B-cell-deficient patients (43). cTfr cells share properties of memory cells and persist for long lasting period *in vivo* and present distinct properties as compared to GC-Tfr cells. Indeed, cTfr cells express less ICOS and present less suppressive functions (66). Upon second immunization, cTfr cells home to GC and suppress Tfh and B cell activation irrespective of their Ag specificity. Therefore, the limited suppressive capacity of cTfr cells may contribute to improve the performance of memory Tfh cell response thereby enabling productive recall Ab responses. Even if the cTfr cells exit the SLO before GC reaction (66, 67), many studies have used the ratio of circulating Tfr and Tfh cells as an indicator of the ongoing GC reaction during autoimmune diseases such as systemic lupus erythematosus (68, 69), multiple sclerosis (70), rheumatoid arthritis (71), and Sjögren's syndrome (72). Other studies also explored the cTfr cell frequency in response to foreign Ag after vaccination or infection. During chronic hepatitis B and

chronic hepatitis C, an increase number of cTfr cells in patients was associated with poor virus eradication and liver injury (73). By contrast, after flu vaccination, cTfr cell frequencies increased and correlated with enhanced anti-flu Ab responses (70). Due to the limited access to SLO organs from humans, the circulating follicular T cells represent an ideal indicator of B cell responses even if the ratio of cTfr/cTfh cells corresponds to a biased marker of GC events. Therefore, a better characterization of cTfr cell development and function during physiological and pathological contexts is required in order to perform an appropriate assumption of the GC reaction.

MULTIFACETED Tfr CELL FUNCTION DURING GC B CELL SELECTION

Tfr Cells Are Negative Regulators

T follicular regulatory cells have the surface profile of Tfh cells (CXCR5^{hi} PD-1^{hi} ICOS⁺) and localize in the GC, but they also express Foxp3 and exhibit a CTLA-4^{hi}, GITR^{hi}, ICOS^{hi}, and IL-10^{hi} phenotype that is the characteristic of activated Treg. CTLA-4 and PD-1 are known to enhance the suppressive activity of Treg (74, 75). Tfr cells express these molecules uniformly, however, CTLA-4 and PD-1 display distinct Tfr cell functions. CTLA-4 deficiency leads to a decrease production of Ag-specific Ab (76) through mechanisms that either alter (77) or not (76) the co-stimulatory signals provided by GC-B cells. By contrast, PD-1 deficiency leads to an increase of the suppressive activity of Tfr cells (61). Importantly, many molecules involved in Tfr cell differentiation are also important in Tfr cell function. As an example, alteration of the mTOR1 signaling pathway in differentiated Tfr cells leads to decreased expression of CTLA-4, ICOS, and PD-1, which consequently leads to a decrease of Tfr cell suppressive activity (54). Tfr cells express many other Treg cell inhibitory molecules such as GITR, granzyme A, and CD103, however, whether these molecules participate to the regulation of Ab production by Tfr cells still remain unknown.

The suppressive function of Tfr cell leads to durable and persistent inhibition of B cells through epigenetic modifications (78). B cells suppressed by Tfr cells decrease expression of genes involved in metabolic pathways and class switch recombination. Tfr cells also suppress genes involved in Tfh cell effector functions such as IL-4 and IL-21. Interestingly, IL-21 produced by Tfh cells overcomes the Tfr-suppressive function by stimulating B cell metabolism and function. IL-21 might alter Tfr cell metabolism and thereby reduces suppressive activity, as has been observed in Treg cells (79, 80). Therefore, IL-21 secretion might be a key factor in balancing the B cell and Tfr cell activity.

While Tfr cells arise from Treg and use many of the Treg attributes to regulate GCs, these cells have also their proper mechanisms. Unexpectedly, it was shown very recently that Tfr cells do not express CD25 (81). IL-2 promotes Treg proliferation (82). By contrast, it was demonstrated that IL-2 inhibits Tfr cell formation after *Influenza* infection (65). Moreover, while the Treg/Tfh cell balance is regulated by the IL-2 axis, the Tfr/Tfh cell balance is regulated by the IL-1 axis (81). Tfh cells express the agonist receptor IL-1R1 that promotes Tfh cell activation in response to

IL-1. By contrast, Tfr cells express the IL-1 decoy receptor IL-1R2 and the IL-1 receptor antagonist IL-1Ra. Consequently, Tfr cells limit Tfh cell activation by limiting IL-1 availability within the GC (81). Thus, the dialog between effector and Treg use appropriate mechanisms depending on its location: IL-2 axis by Treg cells in the T cell zone vs IL-1 axis by Tfr cells in the B cell follicle.

Other Functions of Tfr Cells

Beside the regulatory phenotype of Tfr cells, suppressive machinery during GC B cell selection can provide unexpected positive functions to Tfr cells (see **Table 1**). Several studies have demonstrated a role for Tfr cells in the control of the affinity maturation of B cells in response to foreign Ag (6–8) or within the gut where IgA production allows microbiota diversification (83). However, conflicting results were obtained regarding how Tfr cells control B cell maturation. The most probable reason for this divergence is related to the different experimental systems used to deplete Tfr cell population. One of these strategies was the use of mixed bone marrow chimera of SAP-deficient mice and DEREK mouse model, in which diphtheria toxin (DT) receptor expression is under the control of the Foxp3 promoter. Treatment of these chimeras with DT leads to selective depletion of Tfr cells (7). As SAP is involved during APC/T and T/T cell interactions (84), its absence in Treg may contribute to the modulation of immune responses and expansion of Tfh cells. Other strategies consisted in the co-transfer of naive CD4⁺ T cells with either Bcl6- or CXCR5-deficient Treg into T cell-deficient mice (6, 8, 83). In this context, the lymphopenia of T cell-deficient mice can lead to T cell expansion and therefore may mask the function of Tfr cells in regulating the Tfh cells amplitude. A recent study used a novel mouse model by crossing the Foxp3cre mouse strain with Bcl6floxed mice, which rendered possible the complete depletion of the Tfr cell compartment and left intact the Treg pool (85). Unexpectedly, following immunization with non-self Ag in these animals, Tfr cell deficiency showed no impact on the Tfh and GC B cell magnitude. By contrast, Tfr cell deficiency led to a decreased avidity of Ag-specific Ab, a decrease of Ag-specific IgG and an increase of IgA titers. In the context of autoimmunity using the pristane-induced lupus model in these mice, Tfr cell deficiency led to an increase of anti-dsDNA IgA titers while similar titers of anti-dsDNA IgM and IgG were observed. Finally, absence of Tfr in gut-associated lymphoid tissues led to a decrease of the helper properties of Tfh cells, notably by reducing

IL-21 production (83). Overall, these studies demonstrated that Tfr cells do not only control GC reaction magnitude but can also control the downstream events resulting in class switching and maturation and, ultimately, optimize GC responses by increasing Ab avidity, promoting IgG class switch, repressing abnormal IgA responses, and preventing the production of self-specific Ab. Efficient GC reaction relies on the fine regulation of the processes of somatic hypermutation and class switch recombination during B cell clone proliferation within the dark zone (DZ) and of GC B cell selection in the light zone. These sequential events depend on increasing the surface of entanglement between GC Tfh and GC B cells through co-stimulatory molecules and TCR engagement, limiting the number of GC Tfh cells in order to increase the threshold for GC B cell selection and controlling the cytokine production by GC Tfh cells to orientate the class switching. Therefore, the multifaceted function of Tfr cells may reflect the function of different subsets of Tfr cells (see **Table 2**). This heterogeneity in the Tfr cell compartment could also explain the divergence in the results obtained when studying the impact of Tfr cell deficiency.

One attribute of Tfr cell subdivision could depend on the Tfr cell ontogeny. The Tfr cells deriving from pTreg are mostly specific for the immunizing Ag while only a fraction of Tfr deriving from tTreg shares its Ag specificity with Tfh cells (41). Therefore, the Tfr cells can be stratified into two distinct populations according to their Ag-specificity. The non-Ag-specific Tfr cells could restrict the outgrowth of non-Ag-specific GC B cells while the Ag-specific Tfr cells could control, in a cognate fashion, the extent of the Ag-specific GC B cells. Moreover, the Ag-specific Tfr cells could play a crucial role in the selection mechanisms of high-affinity B cell clones. The competition for T cell help promotes affinity maturation of B cells. It is conceivable that Ag-specific Tfr, as they could compete with GC Tfh cells to interact with pMHCII at the surface of GC B cells, could limit Tfh/B cell interaction and consequently could lead to an increase affinity maturation of B cells. Interestingly, human Tfr cells also display phenotypic diversity, according to PD-1 expressing (86). Indeed, PD-1⁺ and PD-1^{low} Tfr cells were characterized in human mesenteric lymph nodes with high abundance of PD-1^{low} Tfr cells. Sage et al. showed that PD-1-deficient Tfr cells were more suppressive than PD-1-sufficient Tfr cells (61). However, human PD-1⁺ and PD-1^{low} Tfr cells suppress Ab responses *in vitro* with the same efficacy (86). Therefore, PD-1 expression on Tfr cells could have distinct function in human and

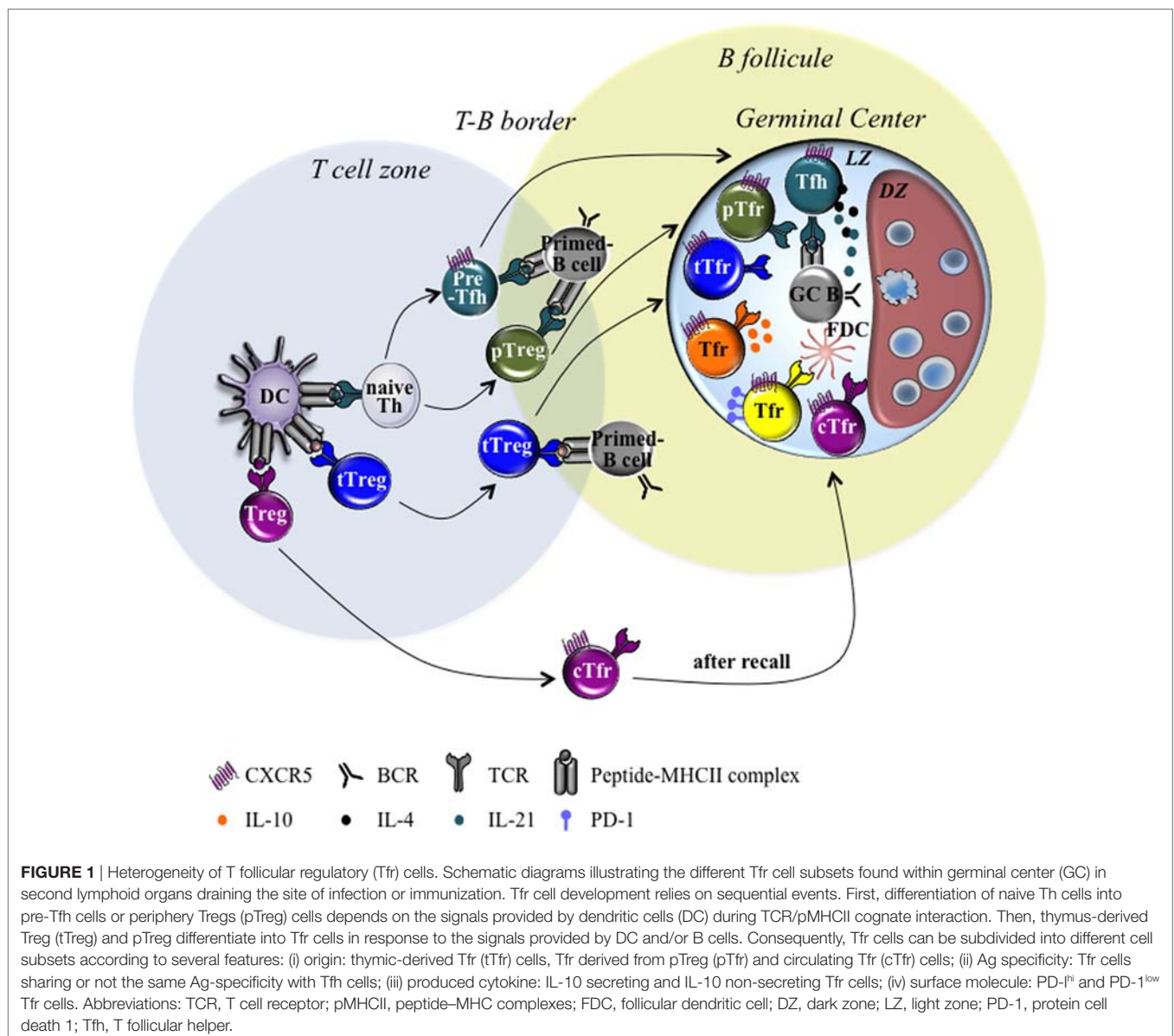
TABLE 2 | List of the different T follicular regulatory (Tfr) cell subsets and their putative functions.

Tfr cell subsets	Putative function	Reference
Tfr cells derived from thymic regulatory T cells	Maintain tolerance and restrain magnitude of GC B cells	(6–8)
Tfr derived from naive Th cells	Mostly specific for the immunizing Ag allowing the control of Ag-specific B cell response through cognate interaction	(41)
IL10-producing Tfr cells	Promote plasmablast differentiation and GC development	(10)
PD-1 ⁺ Tfr cells in mouse and PD-1 ^{lo} in human	Abundant population that displays less suppressive activity preventing excessive suppression of GC reaction	(61, 86)
PD-1 ⁺ Tfr cells in human	Display greater suppressive activity (TGFβ ^{hi} , IL-10 ^{hi} , CTLA-4 ^{hi})	(86)
Circulating Tfr cells	Display less suppressive capacity and home to GC upon recall contributing to faster memory T follicular helper cell response	(66)

mouse. While PD-1 expression decreases the suppressive activity of mouse Tfr cell, PD-1 expression on human Tfr cells could modulate the Abs responses *in vivo*. Indeed, human PD-1⁺ Tfr cells express high levels of CD38, CTLA-4, and GARP, a protein critical for the surface expression of latent TGFβ in activated human Treg cells (87), as compared to PD-1^{low} Tfr cells (86).

T follicular regulatory cell subdivision could also rely on the cytokines produced by these cells, such as IL-10 (10). Indeed, a recent study has shown the ability of Tfr cells not to suppress but to promote GC response through provision of IL-10, an anti-inflammatory cytokine (10). More precisely, specific ablation of IL-10 in Tfr cells only led to a decrease of the GC response. IL-10 was shown to act specifically on B cells and to promote DZ phenotype through the induction of the nuclear factor FOXO1 and to potentially enhance their affinity maturation (10).

IL-10-secreting and non-secreting Tfr cells could represent two distinct populations. Even if the two populations express CTLA-4, they could dialog with distinct cell targets and could have different functional outcomes. Finally, as stated already, cTfr cells emerge before GC reaction and are able to home to GC during secondary response to suppress GC Tfh and B cell activation (66, 67). Therefore, GC reaction that contains cTfr-derived Tfr cells could restrain the generation of new-formed GC-Tfr cells and could display less suppressive activity, which could contribute to improve the performance of memory B cell response. Overall, the Tfr cell population that enters the GC reaction can rely on different features (origin, Ag specificity, cytokine produced, expression of surface molecules, etc.) that could impact the Tfr cell function and, consequently, the quality of the B cell response (see Figure 1).



CONCLUSION

Humoral responses have a pivotal role in the development of protective immune responses. Follicular T cells form the main T cell population controlling GC reaction. Over time, follicular T cells have been broadly characterized and stratified into different specialized subsets providing B cell helper function. More recently, Tfr cells have been identified among the follicular T cells. While, these Tfr cells have been initially described as negative regulators, many new evidence demonstrate that Tfr cells do not act as passive inhibitor but can integrate the environmental cues and achieve adapted program to regulate the corresponding type of immune response within the GC. These proprieties are mandatory to get a correct cell communication within the GC and to guaranty

relevant B cell maturation. Therefore, better characterization of the properties of Tfr cell subsets in regulating GC reaction is mandatory and could allow defining new vaccination strategies through selective modulation of particular Tfr cell subsets.

AUTHOR CONTRIBUTIONS

NF and MA conceived and wrote the manuscript.

FUNDING

This research was funded by Agence Nationale de la Recherche (ANR16-CE15-0002-02, ANR16-CE15-0019-02) and Institut National Du Cancer (INCA PLBIO 2018-060).

REFERENCES

- Shulman Z, Gitlin AD, Weinstein JS, Lainez B, Esplugues E, Flavell RA, et al. Dynamic signaling by T follicular helper cells during germinal center B cell selection. *Science* (2014) 345(6200):1058–62. doi:10.1126/science.1257861
- Vinuesa CG, Linterman MA, Yu D, MacLennan IC. Follicular helper T cells. *Annu Rev Immunol* (2016) 34:335–68. doi:10.1146/annurev-immunol-041015-055605
- Knowlton ZA, Sant AJ. CD4 T cell epitope specificity determines follicular versus non-follicular helper differentiation in the polyclonal response to influenza infection or vaccination. *Sci Rep* (2016) 6:28287. doi:10.1038/srep28287
- Tubo NJ, Pagan AJ, Taylor JJ, Nelson RW, Linehan JL, Ertelt JM, et al. Single naive CD4+ T cells from a diverse repertoire produce different effector cell types during infection. *Cell* (2013) 153(4):785–96. doi:10.1016/j.cell.2013.04.007
- Fazilleau N, McHeyzer-Williams LJ, Rosen H, McHeyzer-Williams MG. The function of follicular helper T cells is regulated by the strength of T cell antigen receptor binding. *Nat Immunol* (2009) 10(4):375–84. doi:10.1038/ni.1704
- Chung Y, Tanaka S, Chu F, Nurieva RI, Martinez GJ, Rawal S, et al. Follicular regulatory T cells expressing Foxp3 and Bcl-6 suppress germinal center reactions. *Nat Med* (2011) 17(8):983–8. doi:10.1038/nm.2426
- Linterman MA, Pierson W, Lee SK, Kallies A, Kawamoto S, Rayner TF, et al. Foxp3+ follicular regulatory T cells control the germinal center response. *Nat Med* (2011) 17(8):975–82. doi:10.1038/nm.2425
- Wollenberg I, Agua-Doce A, Hernández A, Almeida C, Oliveira VG, Faro J, et al. Regulation of the germinal center reaction by Foxp3+ follicular regulatory T cells. *J Immunol* (2011) 187(9):4553–60. doi:10.4049/jimmunol.1101328
- Amé-Thomas P, Le Priol J, Yssel H, Caron G, Pangault C, Jean R, et al. Characterization of intratumoral follicular helper T cells in follicular lymphoma: role in the survival of malignant B cells. *Leukemia* (2012) 26(5):1053–63. doi:10.1038/leu.2011.301
- Laidlaw BJ, Lu Y, Amezquita RA, Weinstein JS, Vander Heiden JA, Gupta NT, et al. Interleukin-10 from CD4(+) follicular regulatory T cells promotes the germinal center response. *Sci Immunol* (2017) 2(16). doi:10.1126/sciimmunol.aan4767
- Fazilleau N, Mark L, McHeyzer-Williams LJ, McHeyzer-Williams MG. Follicular helper T cells: lineage and location. *Immunity* (2009) 30(3):324–35. doi:10.1016/j.immuni.2009.03.003
- King C, Tangye SG, Mackay CR. T follicular helper (TFH) cells in normal and dysregulated immune responses. *Annu Rev Immunol* (2008) 26:741–66. doi:10.1146/annurev.immunol.26.021607.090344
- Chtanova T, Tangye SG, Newton R, Frank N, Hodge MR, Rolph MS, et al. T follicular helper cells express a distinctive transcriptional profile, reflecting their role as non-Th1/Th2 effector cells that provide help for B cells. *J Immunol* (2004) 173(1):68–78. doi:10.4049/jimmunol.173.1.68
- Johnston RJ, Poholek AC, DiToro D, Yusuf I, Eto D, Barnett B, et al. Bcl6 and Blimp-1 are reciprocal and antagonistic regulators of T follicular helper cell differentiation. *Science* (2009) 325(5943):1006–10. doi:10.1126/science.1175870
- Nurieva RI, Chung Y, Martinez GJ, Yang XO, Tanaka S, Matskevitch TD, et al. Bcl6 mediates the development of T follicular helper cells. *Science* (2009) 325(5943):1001–5. doi:10.1126/science.1176676
- Yu D, Rao S, Tsai LM, Lee SK, He Y, Sutcliffe EL, et al. The transcriptional repressor Bcl-6 directs T follicular helper cell lineage commitment. *Immunity* (2009) 31(3):457–68. doi:10.1016/j.immuni.2009.07.002
- Liu X, Yan X, Zhong B, Nurieva RI, Wang A, Wang X, et al. Bcl6 expression specifies the T follicular helper cell program in vivo. *J Exp Med* (2012) 209(10):S1–24. doi:10.1084/jem.20120219
- Liu X, Chen X, Zhong B, Wang A, Wang X, Chu F, et al. Transcription factor achaete-scute homologue 2 initiates follicular T-helper-cell development. *Nature* (2014) 507(7493):513–8. doi:10.1038/nature12910
- Ray JP, Marshall HD, Laidlaw BJ, Starnon MM, Kaech SM, Craft J. Transcription factor STAT3 and type I interferons are corepressive insulators for differentiation of follicular helper and T helper 1 cells. *Immunity* (2014) 40(3):367–77. doi:10.1016/j.immuni.2014.02.005
- Bollig N, Brüstle A, Kellner K, Ackermann W, Abass E, Raifer H, et al. Transcription factor IRF4 determines germinal center formation through follicular T-helper cell differentiation. *Proc Natl Acad Sci U S A* (2012) 109(22):8664–9. doi:10.1073/pnas.1205834109
- Biswas PS, Gupta S, Stirzaker RA, Kumar V, Jessberger R, Lu TT, et al. Dual regulation of IRF4 function in T and B cells is required for the coordination of T-B cell interactions and the prevention of autoimmunity. *J Exp Med* (2012) 209(3):581–96. doi:10.1084/jem.20111195
- Choi YS, Gullicksrud JA, Xing S, Zeng Z, Shan Q, Li F, et al. LEF-1 and TCF-1 orchestrate T(FH) differentiation by regulating differentiation circuits upstream of the transcriptional repressor Bcl6. *Nat Immunol* (2015) 16(9):980–90. doi:10.1038/ni.3226
- Campbell DJ, Kim CH, Butcher EC. Separable effector T cell populations specialized for B cell help or tissue inflammation. *Nat Immunol* (2001) 2(9):876–81. doi:10.1038/ni0901-876
- Nurieva RI, Chung Y, Hwang D, Yang XO, Kang HS, Ma L, et al. Generation of T follicular helper cells is mediated by interleukin-21 but independent of T helper 1, 2, or 17 cell lineages. *Immunity* (2008) 29(1):138–49. doi:10.1016/j.immuni.2008.05.009
- Forster R, Emrich T, Kremmer E, Lipp M. Expression of the G-protein-coupled receptor BLR1 defines mature, recirculating B cells and a subset of T-helper memory cells. *Blood* (1994) 84(3):830–40.
- Morita R, Schmitt N, Benteibibel SE, Ranganathan R, Bourdery L, Zurawski G, et al. Human blood CXCR5(+)CD4(+) T cells are counterparts of T follicular cells and contain specific subsets that differentially support antibody secretion. *Immunity* (2011) 34(1):108–21. doi:10.1016/j.immuni.2010.12.012
- Crotty S. T follicular helper cell differentiation, function, and roles in disease. *Immunity* (2014) 41(4):529–42. doi:10.1016/j.immuni.2014.10.004
- Reinhardt RL, Liang HE, Locksley RM. Cytokine-secreting follicular T cells shape the antibody repertoire. *Nat Immunol* (2009) 10(4):385–93. doi:10.1038/ni.1715

29. Ding Y, Li J, Wu Q, Yang P, Luo B, Xie S, et al. IL-17RA is essential for optimal localization of follicular Th cells in the germinal center light zone to promote autoantibody-producing B cells. *J Immunol* (2013) 191(4):1614–24. doi:10.4049/jimmunol.1300479
30. Glatman Zaretsky A, Taylor JJ, King IL, Marshall FA, Mohrs M, Pearce EJ. T follicular helper cells differentiate from Th2 cells in response to helminth antigens. *J Exp Med* (2009) 206(5):991–9. doi:10.1084/jem.20090303
31. Hirota K, Turner JE, Villa M, Duarte JH, Demengeot J, Steinmetz OM, et al. Plasticity of Th17 cells in Peyer's patches is responsible for the induction of T cell-dependent IgA responses. *Nat Immunol* (2013) 14(4):372–9. doi:10.1038/ni.2552
32. Yusuf I, Kageyama R, Monticelli L, Johnston RJ, Ditoro D, Hansen K, et al. Germinal center T follicular helper cell IL-4 production is dependent on signaling lymphocytic activation molecule receptor (CD150). *J Immunol* (2010) 185(1):190–202. doi:10.4049/jimmunol.0903505
33. Kroenke MA, Eto D, Locci M, Cho M, Davidson T, Haddad EK, et al. Bcl6 and Maf cooperate to instruct human follicular helper CD4 T cell differentiation. *J Immunol* (2012) 188(8):3734–44. doi:10.4049/jimmunol.1103246
34. Ma CS, Suryani S, Avery DT, Chan A, Nanan R, Santner-Nanan B, et al. Early commitment of naive human CD4(+) T cells to the T follicular helper (TFH) cell lineage is induced by IL-12. *Immunol Cell Biol* (2009) 87(8):590–600. doi:10.1038/icb.2009.64
35. Sakaguchi S, Yamaguchi T, Nomura T, Ono M. Regulatory T cells and immune tolerance. *Cell* (2008) 133(5):775–87. doi:10.1016/j.cell.2008.05.009
36. Koch MA, Tucker-Heard G, Perdue NR, Killebrew JR, Urdahl KB, Campbell DJ. The transcription factor T-bet controls regulatory T cell homeostasis and function during type 1 inflammation. *Nat Immunol* (2009) 10(6):595–602. doi:10.1038/ni.1731
37. Zheng Y, Chaudhry A, Kas A, deRoos P, Kim JM, Chu TT, et al. Regulatory T-cell suppressor program co-opts transcription factor IRF4 to control T(H)2 responses. *Nature* (2009) 458(7236):351–6. doi:10.1038/nature07674
38. Chaudhry A, Rudra D, Treuting P, Samstein RM, Liang Y, Kas A, et al. CD4+ regulatory T cells control TH17 responses in a Stat3-dependent manner. *Science* (2009) 326(5955):986–91. doi:10.1126/science.1172702
39. Alexander CM, Tygrett LT, Boyden AW, Wolniak KL, Legge KL, Waldschmidt TJ. T regulatory cells participate in the control of germinal centre reactions. *Immunology* (2011) 133(4):452–68. doi:10.1111/j.1365-2567.2011.03456.x
40. Lim HW, Hillsamer P, Kim CH. Regulatory T cells can migrate to follicles upon T cell activation and suppress GC-Th cells and GC-Th cell-driven B cell responses. *J Clin Invest* (2004) 114(11):1640–9. doi:10.1172/JCI200422325
41. Aloulou M, Carr EJ, Gador M, Bignon A, Liblau RS, Fazilleau N, et al. Follicular regulatory T cells can be specific for the immunizing antigen and derive from naive T cells. *Nat Commun* (2016) 7:10579. doi:10.1038/ncomms10579
42. Maceiras AR, Almeida SCP, Mariotti-Ferrandiz E, Chaara W, Jebbawi F, Six A, et al. T follicular helper and T follicular regulatory cells have different TCR specificity. *Nat Commun* (2017) 8:15067. doi:10.1038/ncomms15067
43. Maceiras AR, Fonseca VR, Agua-Doce A, Graca L. T follicular regulatory cells in mice and men. *Immunology* (2017) 152(1):25–35. doi:10.1111/imm.12774
44. Picca CC, Larkin J 3rd, Boesteanu A, Lerman MA, Rankin AL, Caton AJ. Role of TCR specificity in CD4+ CD25+ regulatory T-cell selection. *Immunol Rev* (2006) 212:74–85. doi:10.1111/j.0105-2896.2006.00416.x
45. Moon JJ, Dash P, Oguin TH 3rd, McClaren JL, Chu HH, Thomas PG, et al. Quantitative impact of thymic selection on Foxp3+ and Foxp3- subsets of self-peptide/MHC class II-specific CD4+ T cells. *Proc Natl Acad Sci U S A* (2011) 108(35):14602–7. doi:10.1073/pnas.1109806108
46. Shafiani S, Dinh C, Ertelt JM, Moguche AO, Siddiqui I, Smigiel KS, et al. Pathogen-specific Treg cells expand early during *Mycobacterium tuberculosis* infection but are later eliminated in response to interleukin-12. *Immunity* (2013) 38(6):1261–70. doi:10.1016/j.immuni.2013.06.003
47. Shafiani S, Tucker-Heard G, Kariyone A, Takatsu K, Urdahl KB. Pathogen-specific regulatory T cells delay the arrival of effector T cells in the lung during early tuberculosis. *J Exp Med* (2010) 207(7):1409–20. doi:10.1084/jem.20091885
48. Fazilleau N, Bachelez H, Gougeon ML, Viguier M. Cutting edge: size and diversity of CD4+CD25high Foxp3+ regulatory T cell repertoire in humans: evidence for similarities and partial overlapping with CD4+CD25- T cells. *J Immunol* (2007) 179(6):3412–6. doi:10.4049/jimmunol.179.6.3412
49. Hsieh CS, Zheng Y, Liang Y, Fontenot JD, Rudensky AY. An intersection between the self-reactive regulatory and nonregulatory T cell receptor repertoires. *Nat Immunol* (2006) 7(4):401–10. doi:10.1038/ni1318
50. Pacholczyk R, Kern J, Singh N, Iwashima M, Kraj P, Ignatowicz L. Nonspecific antigens are the cognate specificities of Foxp3+ regulatory T cells. *Immunity* (2007) 27(3):493–504. doi:10.1016/j.immuni.2007.07.019
51. Betts RJ, Prabhu N, Ho AW, Lew FC, Hutchinson PE, Rotzschke O, et al. Influenza A virus infection results in a robust, antigen-responsive, and widely disseminated Foxp3+ regulatory T cell response. *J Virol* (2012) 86(5):2817–25. doi:10.1128/JVI.05685-11
52. Suffia IJ, Reckling SK, Piccirillo CA, Goldszmid RS, Belkaid Y. Infected site-restricted Foxp3+ natural regulatory T cells are specific for microbial antigens. *J Exp Med* (2006) 203(3):777–88. doi:10.1084/jem.20052056
53. Kieback E, Hilgenberg E, Stervbo U, Lampropoulou V, Shen P, Bunse M, et al. Thymus-derived regulatory T cells are positively selected on natural self-antigen through cognate interactions of high functional avidity. *Immunity* (2016) 44(5):1114–26. doi:10.1016/j.immuni.2016.04.018
54. Xu L, Huang Q, Wang H, Hao Y, Bai Q, Hu J, et al. The kinase mTORC1 promotes the generation and suppressive function of follicular regulatory T cells. *Immunity* (2017) 47(3):538–51.e5. doi:10.1016/j.immuni.2017.08.011
55. Vaeth M, Müller G, Stauss D, Dietz L, Klein-Hessling S, Serfling E, et al. Follicular regulatory T cells control humoral autoimmunity via NFAT2-regulated CXCR5 expression. *J Exp Med* (2014) 211(3):545–61. doi:10.1084/jem.20130604
56. Yang J, Lin X, Pan Y, Wang J, Chen P, Huang H, et al. Critical roles of mTOR complex 1 and 2 for T follicular helper cell differentiation and germinal center responses. *Elife* (2016) 5:e17936. doi:10.7554/eLife.17936
57. Zeng H, Cohen S, Guy C, Shrestha S, Neale G, Brown SA, et al. mTORC1 and mTORC2 kinase signaling and glucose metabolism drive follicular helper T cell differentiation. *Immunity* (2016) 45(3):540–54. doi:10.1016/j.immuni.2016.08.017
58. Linterman MA, Denton AE, Divekar DP, Zvetkova I, Kane L, Ferreira C, et al. CD28 expression is required after T cell priming for helper T cell responses and protective immunity to infection. *Elife* (2014) 3:e03180. doi:10.7554/eLife.03180
59. Qi H, Cannons JL, Klauschen F, Schwartzberg PL, Germain RN. SAP-controlled T-B cell interactions underlie germinal centre formation. *Nature* (2008) 455(7214):764–9. doi:10.1038/nature07345
60. Leavenworth JW, Verbinen B, Yin J, Huang H, Cantor H. A p85alpha-osteopontin axis couples the receptor ICOS to sustained Bcl-6 expression by follicular helper and regulatory T cells. *Nat Immunol* (2015) 16(1):96–106. doi:10.1038/ni.3050
61. Sage PT, Francisco LM, Carman CV, Sharpe AH. The receptor PD-1 controls follicular regulatory T cells in the lymph nodes and blood. *Nat Immunol* (2013) 14(2):152–61. doi:10.1038/ni.2496
62. Miyazaki M, Miyazaki K, Chen S, Itoi M, Miller M, Lu LF, et al. Id2 and Id3 maintain the regulatory T cell pool to suppress inflammatory disease. *Nat Immunol* (2014) 15(8):767–76. doi:10.1038/ni.2928
63. Johnston RJ, Choi YS, Diamond JA, Yang JA, Crotty S. STAT5 is a potent negative regulator of TFH cell differentiation. *J Exp Med* (2012) 209(2):243–50. doi:10.1084/jem.20111174
64. Jandl C, Liu SM, Cañete PF, Warren J, Hughes WE, Vogelzang A, et al. IL-21 restricts T follicular regulatory T cell proliferation through Bcl-6 mediated inhibition of responsiveness to IL-2. *Nat Commun* (2017) 8:14647. doi:10.1038/ncomms14647
65. Botta D, Fuller MJ, Marquez-Lago TT, Bachus H, Bradley JE, Weinmann AS, et al. Dynamic regulation of T follicular regulatory cell responses by interleukin 2 during influenza infection. *Nat Immunol* (2017) 18(11):1249–60. doi:10.1038/ni.3837
66. Sage PT, Alvarez D, Godec J, von Andrian UH, Sharpe AH. Circulating T follicular regulatory and helper cells have memory-like properties. *J Clin Invest* (2014) 124(12):5191–204. doi:10.1172/JCI76861
67. Fonseca VR, Agua-Doce A, Maceiras AR, Pierson W, Ribeiro F, Romão VC, et al. Human blood Tfr cells are indicators of ongoing humoral activity not fully licensed with suppressive function. *Sci Immunol* (2017) 2(14). doi:10.1126/sciimmunol.aan1487
68. Liu C, Wang D, Song Y, Lu S, Zhao J, Wang H. Increased circulating CD4(+) CXCR5(+)FoxP3(+) follicular regulatory T cells correlated with severity of

- systemic lupus erythematosus patients. *Int Immunopharmacol* (2018) 56:261–8. doi:10.1016/j.intimp.2018.01.038
69. Xu B, Wang S, Zhou M, Huang Y, Fu R, Guo C, et al. The ratio of circulating follicular T helper cell to follicular T regulatory cell is correlated with disease activity in systemic lupus erythematosus. *Clin Immunol* (2017) 183:46–53. doi:10.1016/j.clim.2017.07.004
 70. Dhaeze T, Peelen E, Hombrouck A, Peeters L, Van Wijmeersch B, Lemkens N, et al. Circulating follicular regulatory T cells are defective in multiple sclerosis. *J Immunol* (2015) 195(3):832–40. doi:10.4049/jimmunol.1500759
 71. Liu C, Wang D, Lu S, Xu Q, Zhao L, Zhao J, et al. Increased circulating follicular Treg cells are associated with lower levels of autoantibodies in patients with rheumatoid arthritis in stable remission. *Arthritis Rheumatol* (2018). doi:10.1002/art.40430
 72. Fonseca VR, Romao VC, Agua-Doce A, Santos M, Lopez-Presa D, Ferreira AC, et al. The ratio of blood T follicular regulatory cells to T follicular helper cells marks ectopic lymphoid structure formation while activated follicular helper T cells indicate disease activity in primary Sjogren's syndrome. *Arthritis Rheumatol* (2018) 70(5):774–84. doi:10.1002/art.40424
 73. Wang L, Qiu J, Yu L, Hu X, Zhao P, Jiang Y. Increased numbers of CD5+CD19+CD1dhighIL-10+ Bregs, CD4+Foxp3+ Tregs, CD4+CXCR5+Foxp3+ follicular regulatory T (TFR) cells in CHB or CHC patients. *J Transl Med* (2014) 12:251. doi:10.1186/s12967-014-0251-9
 74. Curran MA, Montalvo W, Yagita H, Allison JP. PD-1 and CTLA-4 combination blockade expands infiltrating T cells and reduces regulatory T and myeloid cells within B16 melanoma tumors. *Proc Natl Acad Sci U S A* (2010) 107(9):4275–80. doi:10.1073/pnas.0915174107
 75. Wang W, Lau R, Yu D, Zhu W, Korman A, Weber J. PD1 blockade reverses the suppression of melanoma antigen-specific CTL by CD4+ CD25(Hi) regulatory T cells. *Int Immunol* (2009) 21(9):1065–77. doi:10.1093/intimm/dxp072
 76. Sage PT, Paterson AM, Lovitch SB, Sharpe AH. The coinhibitory receptor ctla-4 controls B cell responses by modulating T follicular helper, T follicular regulatory, and T regulatory cells. *Immunity* (2014) 41(6):1026–39. doi:10.1016/j.immuni.2014.12.005
 77. Wing JB, Ise W, Kurosaki T, Sakaguchi S. Regulatory T cells control antigen-specific expansion of Tfh cell number and humoral immune responses via the coreceptor CTLA-4. *Immunity* (2014) 41(6):1013–25. doi:10.1016/j.immuni.2014.12.006
 78. Sage PT, Ron-Harel N, Juneja VR, Sen DR, Maleri S, Sunghak W, et al. Suppression by TFR cells leads to durable and selective inhibition of B cell effector function. *Nat Immunol* (2016) 17(12):1436–46. doi:10.1038/ni.3578
 79. Fabrizi M, Marchetti V, Mavilio M, Marino A, Casagrande V, Cavallera M, et al. IL-21 is a major negative regulator of IRF4-dependent lipolysis affecting Tregs in adipose tissue and systemic insulin sensitivity. *Diabetes* (2014) 63(6):2086–96. doi:10.2337/db13-0939
 80. Peluso I, Fantini MC, Fina D, Caruso R, Boirivant M, MacDonald TT, et al. IL-21 counteracts the regulatory T cell-mediated suppression of human CD4+ T lymphocytes. *J Immunol* (2007) 178(2):732–9. doi:10.4049/jimmunol.178.2.732
 81. Ritvo PG, Churlaud G, Quiniou V, Florez L, Brimaud F, Fourcade G, et al. Tfr cells lack IL-2Ralpha but express decoy IL-1R2 and IL-1Ra and suppress the IL-1-dependent activation of Tfh cells. *Sci Immunol* (2017) 2(15). doi:10.1126/sciimmunol.aan0368
 82. Fontenot JD, Rasmussen JP, Gavin MA, Rudensky AY. A function for interleukin 2 in Foxp3-expressing regulatory T cells. *Nat Immunol* (2005) 6(11):1142–51. doi:10.1038/ni1263
 83. Kawamoto S, Maruya M, Kato LM, Suda W, Atarashi K, Doi Y, et al. Foxp3(+) T cells regulate immunoglobulin a selection and facilitate diversification of bacterial species responsible for immune homeostasis. *Immunity* (2014) 41(1):152–65. doi:10.1016/j.immuni.2014.05.016
 84. Detre C, Keszei M, Romero X, Tsokos GC, Terhorst C. SLAM family receptors and the SLAM-associated protein (SAP) modulate T cell functions. *Semin Immunopathol* (2010) 32(2):157–71. doi:10.1007/s00281-009-0193-0
 85. Wu H, Chen Y, Liu H, Xu LL, Teuscher P, Wang S, et al. Follicular regulatory T cells repress cytokine production by follicular helper T cells and optimize IgG responses in mice. *Eur J Immunol* (2016) 46(5):1152–61. doi:10.1002/eji.201546094
 86. Sayin I, Radtke AJ, Vella LA, Jin W, Wherry EJ, Buggert M, et al. Spatial distribution and function of T follicular regulatory cells in human lymph nodes. *J Exp Med* (2018). doi:10.1084/jem.20171940
 87. Tran DQ, Andersson J, Wang R, Ramsey H, Unutmaz D, Shevach EM. GARP (LRRC32) is essential for the surface expression of latent TGF-beta on platelets and activated FOXP3+ regulatory T cells. *Proc Natl Acad Sci U S A* (2009) 106(32):13445–50. doi:10.1073/pnas.0901944106

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2018 Fazilleau and Aloulou. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



Synaptic Interactions in Germinal Centers

Ilania Papa and Carola G. Vinuesa*

John Curtin School of Medical Research, Australian National University, Acton, ACT, Australia

OPEN ACCESS

Edited by:

Shahram Salek-Ardakani,
Pfizer, United States

Reviewed by:

Jason G. Cyster,
University of California, San
Francisco, United States
Deepak Rao,
Harvard Medical School,
United States

*Correspondence:

Carola G. Vinuesa
carola.vinuesa@anu.edu.au

Specialty section:

This article was submitted to
T Cell Biology,
a section of the journal
Frontiers in Immunology

Received: 24 June 2018

Accepted: 27 July 2018

Published: 13 August 2018

Citation:

Papa I and Vinuesa CG (2018)
Synaptic Interactions in
Germinal Centers.
Front. Immunol. 9:1858.
doi: 10.3389/fimmu.2018.01858

The germinal center (GC) is a complex, highly dynamic microanatomical niche that allows the generation of high-affinity antibody-producing plasma cells and memory B cells. These cells constitute the basis of long-lived highly protective antibody responses. For affinity maturation to occur, B cells undergo multiple rounds of proliferation and mutation of the genes that encode the immunoglobulin V region followed by selection by specialized T cells called follicular helper T (T_{FH}) cells. In order to achieve this result, the GC requires spatially and temporally coordinated interactions between the different cell types, including B and T lymphocytes and follicular dendritic cells. Cognate interactions between T_{FH} and GC B cells resemble cellular connections and synaptic communication within the nervous system, which allow signals to be transduced rapidly and effectively across the synaptic cleft. Such immunological synapses are particularly critical in the GC where the speed of T–B cell interactions is faster and their duration shorter than at other sites. In addition, the antigen-based specificity of cognate interactions in GCs is critical for affinity-based selection in which B cells compete for T cell help so that rapid modulation of the signaling threshold determines the outcome of the interaction. In the context of GCs, which contain large numbers of cells in a highly compacted structure, focused delivery of signals across the interacting cells becomes particularly important. Promiscuous or bystander delivery of positive selection signals could potentially lead to the appearance of long-lived self-reactive B cell clones. Cytokines, cytotoxic granules, and more recently neurotransmitters have been shown to be transferred from T_{FH} to B cells upon cognate interactions. This review describes the current knowledge on immunological synapses occurring during GC responses including the type of granules, their content, and function in T_{FH} -mediated help to B cells.

Keywords: T follicular helper (T_{FH}) cell, germinal center, germinal centre B cells, immunological synapse, dense core granules

IMMUNE SYNAPSE: PRINCIPLES, ORGANIZATION, AND STRUCTURE

The term synapse was first used to describe the typical neural connections in the nervous system, which allow transmission of an electrical or chemical signal from one neuron to a responding cell in close physical contact. Immunologists then co-opted the term and referred to “immunological synapse” to describe the interactions between an antigen-presenting cell (APC) and an antigen-receptor expressing immune cell that involve close contact and the release of molecules such as cytokines across the synaptic space (1, 2).

When naïve T cells recognize peptide-MHC on APCs *via* their TCRs, the TCRs become organized into structures of ~500 nm known as microclusters (MCs). These MCs are more efficient in the

recruitment of kinases and adapters that can initiate an activation signaling cascade (3). During formation of the immunological synapse, the TCR-MCs localize at the center of the interface between the T cells and the APC giving rise to the central supramolecular activation cluster (cSMAC) (4–7). This cSMAC is also called the bull's eye-type immunological synapse, due to its characteristic appearance, as first described by Kupfer (8). The immunological synapse between a T cell and an APC requires close juxtaposition of the membranes from the two different cell types. This is facilitated by a kinetic segregation of molecules that excludes negative regulatory phosphatases such as CD45 that relocates to the most external region or distal SMAC, and allows concentration of the key TCR signaling molecules at the center. This segregation process has been suggested to be an integral part of immune synapse function (9).

Besides TCR signaling, integrins play a key role in T cell activation facilitating the formation of conjugates between T cells and APCs. Lymphocyte function-associated antigen-1 (LFA-1) is one of the most important integrins during the process of T cell activation. LFA-1 and its high-affinity ligand intercellular adhesion molecule 1 (ICAM-1), localize outside of the cSMAC, at the peripheral SMAC (pSMAC). The inside-out signal from TCR or chemokine stimulation elicits conformational changes in LFA-1 that increase affinity for its ligands and therefore adhesion between the interacting cells (10). Binding of LFA-1 by ICAM-1, then leads to what is known as “outside-in” signaling, which contributes to many aspects of T cell activation.

Most membrane-proximal signaling molecules crucial for T cell activation such as ZAP70, LAT, SLP76, PLC- γ , etc., are recruited to TCR-MCs. Regulation of these large protein-complexes determines the outcome of T cell activation, not just in terms of TCR signaling strength but also with regards to the nature of the resulting effector cells (7, 11). It is still unclear how different activation, differentiation, and survival outcomes can derive from changes in the signal strength downstream of these signaling complexes.

Together with T-cell antigen receptors and integrins, two additional groups of receptors are located at the synapse: adhesion and costimulatory receptors. Adhesion is mediated by heterophilic interactions between the signaling lymphocyte activation molecules (SLAM) family members CD2 (expressed on T cells) and CD58 (expressed on APCs). These CD2–CD58 interactions can contribute to TCR signaling processes even when direct TCR stimulation is absent (12).

It has been known for over two decades that costimulatory receptors are poor in eliciting activation signals or inducing cell adhesion on their own, but when combined with signals from other receptors, most prominently the TCR, they can potently enhance T cell activation, adhesion, and differentiation (13–15). The typical T cell costimulator is CD28, a member of the Ig superfamily characterized by a homodimeric structure and a cytoplasmic domain. The cytoplasmic domain of CD28 recruits and activates Lck, which can then phosphorylate and activates protein kinase C (PKC)- θ . In T cells PKC- θ , a critical PKC isoform, contributes to the activation of NF- κ B transcription factors and promotes IL-2 production (16). Ligation of B7-1 (CD80) and B7-2 (CD86) on APCs and interaction

within an immunological synapse regulate CD28 activity (17). Upregulation of CD80 and CD86 on DCs is a downstream effect of toll-like receptors signals and inflammatory cytokines (18, 19). In addition, expression of the inducible T cell costimulator, ICOS on activated T cells helps recruitment of the p50 α PI3K regulatory subunit to the immunological synapse, resulting in stronger activation of PI3K (20).

B CELL–FOLLICULAR DENDRITIC CELL (FDC) SYNAPSES

Synaptic interactions between B cells and FDCs are key for B cells to efficiently extract antigen held in the form of immune complexes on FDCs, and to promote B cell survival until T_{FH} selection and survival signals are delivered. Only those germinal center (GC) B cells able of binding and taking up antigen from FDCs to then present processed peptide to T_{FH} cells can survive and differentiate into memory B cells or plasma cells. Immune complexes in association with activated complement components are bound by immunoglobulin receptors, CD35 and CD21 expressed on FDCs. Interaction between the BCR and antigen held in these immune complexes induces BCR signaling, BCR–antigen MC formation followed by the formation of a mature immune synapse and antigen internalization for subsequent processing and presentation to T cells (21–23).

In vitro studies suggested that GC B cells form unique synaptic structures compared to other B cell subsets (24). GC B cells form synapses containing less antigen than naive B cells, and the antigen is confined preferentially to the pSMAC rather than being localized at the cSMAC. In addition, some GC B cells form small synapses using their characteristic and previously described lamellipodia-like protrusions (24–26).

The early observation that B cells can acquire antigen that is bound to a surface suggested that mechanical forces are required for this process (21). Subsequently, it has been demonstrated that B cells are able to pull and internalize antigens (24), this process is dependent on the nature of the antigen nature and on the physical characteristics of antigen presentation (27). Stiff substrates, such as the FDC membrane, allow higher affinity discrimination, whereas antigen extraction from flexible substrates is more efficient. These findings, together with the observation that GC B cells require stronger forces in order to pull and take up antigen from the synaptic interface compared to naive B cells (24), support the importance of synaptic interactions between FDCs and GC B cells in affinity maturation and antibody production.

The synaptic interactions between GC B cells and FDCs involve several molecules (**Figure 1**). The first group comprises adhesion proteins such as B cell expressed LFA-1 that interacts with ICAM-1 on FDCs, and very-late activation antigen 4 (VLA-4) that interacts with VCAM-1 (28). These molecules do not deliver directly anti-apoptotic signals, but promote the anti-apoptotic functions of FDCs by augmenting cell–cell contact. Of note, GC B cells undergo apoptosis when separated from FDCs.

Expression of VCAM-1 and ICAM-1 on FDCs relies on NF- κ B signaling (29) downstream of FC γ RIIB, as Fc γ 2b^{−/−} mice

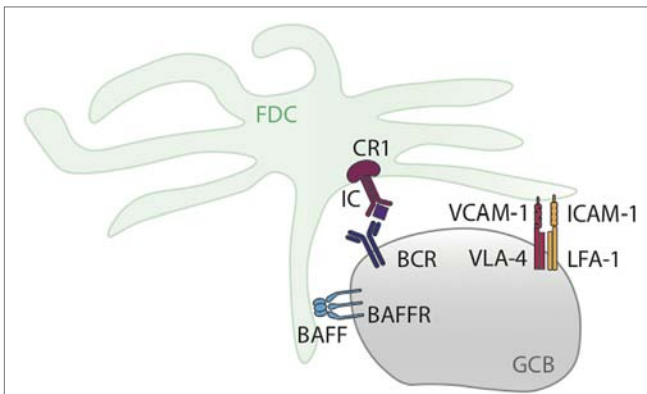


FIGURE 1 | Graphical representation of GC B cell-follicular dendritic cell (FDC) interaction in the germinal center (GC). The diagram shows key molecules involved in the interactions between GC B cells and FDCs happening during GC responses and discussed in Section “B Cell–Follicular Dendritic Cell (FDC) Synapses.”

fail to upregulate *Icam1* or *Vcam1* mRNA and protein on FDCs after immunocomplex formation (30). In mice lacking NF- κ B signaling in FDCs, GCs were smaller and contained more apoptotic cells. Also primary responses to sheep red blood cells were partially reduced and secondary immunizations did not induce strong responses (29), although this could be due to other processes rather than a direct effect of ICAM-1/VCAM-1 regulation.

In vitro studies have suggested that the integrins ICAM-1 and VCAM-1 may play a role in GC B cell survival. Indeed, Lindhout et al. showed that direct interaction between FDCs and human GC B cells facilitated GC B cell survival in culture (31). Subsequent studies revealed that equivalent survival could be achieved when replacing FDCs by coating VCAM-1 and ICAM-1 to the culture plates (28). Rapid death of GC B cells has been shown after diphtheria toxin-mediated ablation of FDCs *in vivo*, supporting a role of FDCs in promoting GC B cell viability (32). It has also been shown *in vivo* that mutation in DOCK8, member of a family of proteins critical for the activation of the Rho family of small GTPases, lead to disrupted concentration of ICAM-1 on B cells forming immune synapses, affecting their survival and affinity maturation (33). Nevertheless, a separate study showed minimal impact of ICAM1 and VCAM1 loss from FDCs on the magnitude of the GC response *in vivo* (34), which questions the importance of FDC-expressed VCAM1 and ICAM1 in GC B cell survival. It is possible that these interactions play a more significant role under some immunization conditions and may differentially impact the plasma cell or memory B cell response.

The second group of molecules influencing FDC–GC B cell interactions comprises pro-survival molecules and growth factors. The B cell survival factor BAFF—also known as BLyS—is produced at high amounts by activated FDCs. Despite its well-described role in the survival of peripheral B cells, it is still unclear whether BAFF plays a role in GC B cell survival. *In vivo* studies describing GC formation with relatively preserved affinity maturation in BAFF-deficient mice suggest it is dispensable (35–37). BAFF/BAFF-R signaling does, however, appear to have a function in GC maintenance, as in the absence of this factor,

GCs disappear a few days after their formation. Lack of BAFF also leads to failure of FDC maturation and as a consequence, stimulation of B cells through immune complexes cannot occur (35, 37). It is plausible this FDC phenotype may be an indirect effect of B cell lymphopenia in the absence of BAFF. In BAFF-R defective A/WySnJ mice, the FDC reticulum was normal and GCs could form, but proliferation of GC B cells was impaired (35). BAFF also signals through the receptors TACI and BCMA and mice deficient in these receptors displayed normal GC formation (38, 39) suggesting BAFF-R signaling is responsible for the observed effects of BAFF on GC B cells. However, some B cells in A/WySnJ mice (lacking BAFF-R) are able to mature, enter the GC reaction and support FDC maturation, whereas B cells from BAFF-deficient mice cannot (40), suggesting that in the absence of BAFF-R, the other receptors for BAFF and/or APRIL (BCMA or TACI) may take over the B cell maturation function of BAFF-R, although they do not play an essential role in GC maintenance.

Besides the production of survival factors, FDCs are known for their expression of transmembrane molecules such as the transcobalamin receptor 8D6, and cytokines including IL-6 and IL-15 (41, 42). These surface-expressed or secreted products can also participate in the growth of GC B-cells. 8D6, also known as or CD320 can promote GC B cell growth. 8D6 also appears to support the proliferation of plasma cell precursors generated by IL-10, enhancing antibody secretion (42, 43). Human FDCs also produce IL-15, and *in vitro* its membrane-bound form has been shown to signal through IL-2/IL-15R β (44, 45) to enhance proliferation of GC B cells. This proliferative effect has not been observed for IL-6, although this cytokine is required for proper GC formation (46). It is possible, however, that the effect of IL-6 on GC B cells is indirect, through its well-demonstrated role in promoting T_{FH} cell induction and maintenance (47, 48).

Besides their effects on GC B cells, FDCs also regulate T_{FH} cells. FDC-derived IL-6 has also been suggested to be important for T_{FH} cell maintenance (47). Interactions between T_{FH}-expressed TIGIT (49, 50) and its receptor on FDCs—the high-affinity poliovirus receptor (CD155) (49, 50) may also contribute to T_{FH} regulation by FDCs during thymus-dependent (TD) B cell responses. cT_{FH} TIGIT⁺ cells have been shown to exhibit strong B cell helper functions, inducing plasma cell differentiation and immunoglobulin production (51). Engagement of TIGIT by CD155 promotes IL-10 while it restrains IL-12 production by DCs, leading to reduced T cell activation *in vitro* (52).

T_{FH}–GC B SYNAPSES

Synaptic interactions between T_{FH} and GC B cells are essential for GC formation and GC B cell selection as they facilitate the delivery of T cell-derived helper molecules. Formation of optimal T_{FH}–GC B cell synapses depends on specific interactions between the receptor:ligand pairs described below and summarized in Figure 2.

ICAM-1:LFA-1

Intercellular adhesion molecule 1 also known as cluster of differentiation 54 is a ligand for the integrin LFA-1 and this pair forms

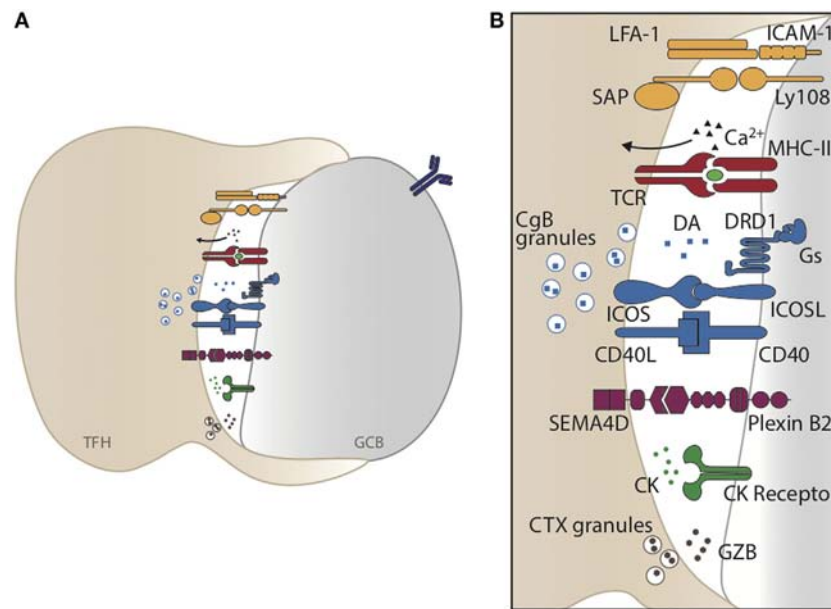


FIGURE 2 | T_H-germinal center (GC) B cell synaptic interaction. **(A)** Graphical representation of T_H and GC B cell interaction. Formation of optimal T_H-GC B cell synapses depends on specific interactions between receptor:ligand pairs and integrins, leading to a specific and focused transmission of cytokines, neurotransmitters, and cytotoxic granules. **(B)** Enlarged representation of the synaptic cleft forming upon T_H-GC B cell interaction.

the peripheral ring (pSMAC) of immune synapses. As mentioned above, during T cell-APC synapses, TCR activation upon binding peptide-MHC II molecules leads to a conformational change in LFA-1 and clustering of LFA-molecules. These structural and positional changes are critical to increase the affinity for ICAM molecules *via* the formation of multivalent associations (53). LFA-1 binding to ICAMs was also shown to be critical for adequate T cell activation and differentiation into effector cells (54, 55).

T_H cells express high levels of LFA-1 (56) and Ag-specific B cells express higher levels of ICAM-1 compared to non Ag-specific cells (57). In addition to cognate pMHCII:TCR interactions, adhesive mechanisms and costimulatory receptors are critical for facilitating T-B conjugate formation and optimal TCR activation (54, 55, 58). Recently Zaretsky and colleagues showed that expression of two LFA-1 ligands on B cells—ICAM-1 and ICAM-2—was required to form the stable and lasting antigen-driven T_H-GC B cell interactions that promote B cell selection. Thus, the B cell antibody response to protein antigens depends on B cell ICAMs for optimal selection by T_H cells. High levels of antigen can sustain short T-B interactions, however, optimal long-lasting contacts are strictly ICAM-dependent (57).

CELL SURFACE-POLARIZED STIMULATORY MOLECULES

CD40:CD40L

T_H-GC B cell contacts require interactions between several key molecules. Among those, CD40L:CD40 interactions play crucial roles within GCs. Consequences of defective CD40-CD40L

signaling highlight their important role in initiation and maintenance of GC responses. Blocking CD40 signals in established GCs leads to complete and rapid GC dissolution in mice (59). Indeed CD40 is critical for GC maintenance and enables light zone (LZ) cells to recycle back to the dark zone and sustain the GC response. In humans, CD40 deficiency causes hyper-IgM syndrome characterized by lack of switched memory B cells and switched serum immunoglobulins. CD40L was also shown to provide survival signals to human GC B cells (60). In mice, CD40 signals also induce ICOSL expression on GC B cells (61).

CD40L is found preformed in GC T cells (62) and is rapidly and transiently relocated on the T cell surface upon TCR ligation. There is recent evidence in mice that ICOS signals can contribute to CD40L upregulation. Indeed, as described above ICOSL signals delivered by GC B cells support the transient but extensive “entanglement” with T_H cells, which in turn stimulates the increased expression of CD40L on the surface of T_H cells (61). CD40L-mediated upregulation of ICOSL expression by GC B cells, followed by ICOSL induction of further CD40L expression on T_H cells has been described as a feed-forward loop that enables high-affinity B cells to repeatedly acquire more T cell help than their lower affinity competitors (61).

Outside GCs it has been shown that both Th1 and Th2 cells transfer CD40L to B cells in an antigen-specific manner (63). Although cultured Th1 and Th2 cells have distinctive immunological synapse structures (64) with Th1 displaying a classical immune synapse and Th2 a multifocal synapse structure, both cell types are equally efficient in antigen-specific T-B interaction (63). Future analysis will be required to confirm the immunological synapse organization of primary Th1 and Th2 cells. In human primary T_H cells, CD40L accumulated in the cSMAC

on supported lipid bilayers containing CD40 and ICOSL (65), suggesting that T_{FH} -GC B cell synapses display the structure of a classical immunological synapse.

SLAM Family Members and SLAM-Associated Protein (SAP)

SLAM-associated protein expression in T cells has been shown to be important for adhesive cognate T-B cell interactions. In the absence of SAP, T-B interactions were less stable and were not long-lasting, impairing T_{FH} differentiation. This stabilization was shown to be a consequence of T-cell intrinsic signaling of SAP, which serves as an adaptor for the SLAM family of immune receptors at the cell-cell interface. B cells express high levels of several SLAM family members including SLAM, CD84, Ly9, and Ly108 molecules. These same molecules are also found in substantial amounts on the surface of activated T cells and in T_{FH} cells (66–70). Most SLAM family members bind in a homophilic fashion to the same molecule expressed on the interacting cell. SLAM molecules recruit SAP to their cytoplasmic tail leading to the downstream signaling cascade required to induce stable adhesive interactions (67, 71). *In vitro* experiments have proposed that CD84 and Ly108 act together during T-B cell synaptic interactions to promote the T_{FH} cell phenotype (66). SAP act as a break to Ly108-mediated recruitment of the inhibitory phosphatase SHP-1 to the T cell synapse. SHP-1 dephosphorylates immunotyrosine switch motifs normally bound by SAP, to limit T-B cell adhesion and prevent formation of sustained T-B cell synaptic interactions. The need of stable T-B cell interactions for an efficient GC response and production of disease-inducing autoantibodies has been also described in the context of autoimmunity (72). It is important to note that the role of SAP/SLAM in stabilizing cognate interactions between T cells and APCs appears to be essential in achieving sustained cognate T-B interactions, but not for T:DC interactions, which are more dependent on integrins (66).

T cell costimulatory ligands expressed by B cells also play key roles in T-B interactions and the resulting proliferation and differentiation of both T and B cells. CD80 and CD86 are markers of B cell activation and function providing important costimulatory signals *via* CD28 that promote T cell proliferation and cytokine production. CD86 is expressed by LZ B cells and promotes the APC capability of B cells (73). Reducing the availability of CD86 molecule by the injection of a blocking antibody (GL-1) during primary responses to NP-CGG resulted in the reduction of serum Ab titers, either IgM or IgG (59) whereas IL-21-mediated sustained elevation of CD86 augmented the magnitude of CD4 T cell responses both *in vitro* and *in vivo* (74). CTLA-4, which is found at high amounts on regulatory cells and some follicular T cells, can also bind to and transendocytose CD86 (75) but its function in GC reactions is still not clear.

Plexin B2 (PlxnB2), Ephrin B1, and BASP1

Plexins constitute a family of transmembrane receptors for semaphorins and regulate multiple processes including synapse formation and axon guidance in the nervous system (76).

PlxnB2 is expressed in the central nervous system upon binding semaphorins promotes axon guidance and migration (77). In the immune system, GC B cells formed in the context of TD but not thymus-independent responses express high amounts of PlxnB2 (25, 26). Recent studies have revealed that PlxnB2 expressed by GC B cells is sensed by Semaphorin 4C (Sema4C) expressed on T_{FH} cells. This PlxnB2-Sema4C interaction promotes T-B adhesion in an antigen-independent manner and guides T_{FH} cell recruitment to the GC (78). For this, PlxnB2 expressed on GC B cells promotes T_{FH} migration from the T zone to the border of the GC. Once positioned at the GC edge, T_{FH} cells have easier access into the GC. The evidence that this positioning is the critical first step comes from the observation that in mice lacking B cell expressed PlxnB2, T_{FH} cells accumulate at the edge of the GC. In these mice, GC T-B interactions are diminished resulting in poor GC-derived antibody responses, including the production of high-affinity antibodies and long-lived plasma cells (78).

In the GC, members of the Ephrin receptors family and their ligands also regulate cell migration and cell-to-cell interaction. Ephrin type-B receptor 4 (EPHB4) and EPHB6 are expressed on T_{FH} cells (79) and one of their ligands, Ephrin-B1 (EFNB1) has been found on GC B cells (79, 80). Ephrin ligands and their receptors are membrane-bound proteins that require direct cell-cell interaction to bind and activate downstream signaling pathways. EFNB1 suppresses GC B- T_{FH} cell adhesion, and mice lacking its expression showed reduction in plasma cell production and accumulation of IL-21-deficient T follicular helper cells within the GC (79). Intriguingly, EPNB1 has been reported to be expressed in a subset of GC B cells that share phenotypic features with memory B cells and are preferentially located in the LZ and outer areas of GCs. It is therefore possible that Ephrin interactions also regulate T_{FH} -B cell interactions leading to memory B cell formation from GC B cells (80).

BASP1 is a myristoylated protein highly expressed in the brain and localized at the inner surface of the plasma membrane in presynaptic neurons. In planar lipid bilayers BASP1 can exert ion channel activity (81). In neurons, BASP1 mediates neurite outgrowth and axonal repair (82). BASP1 expression is absent on resting murine splenic B cells, but can be induced by B-cell activation with anti-IgM and anti-CD40 and is again selectively and strongly upregulated in TD GC B cells. BASP1 is therefore likely to control synaptic processes in GC B cells.

TRANSMITTED MOLECULES

Delivery of Cytokines Across Immune Synapses

One of the major roles of both neurological and immunological synapses is the focused secretion of soluble components into the synaptic cleft where the secreted factors can achieve the desired concentration and selectively act on the precise post-synaptic neuron or antigen-specific lymphoid cell (83–85).

There is also increasing evidence of transfer of cellular contents from T cells to APCs in the form of microvesicles. This is a highly dynamic process by which microvesicles formed

within the T cell are then released into the synaptic interface and taken up by APCs. Within APCs, the contents of microvesicles, which include proteins, RNAs, and microRNAs, can influence gene expression and activation of early signaling pathways (86, 87). Transfer in the opposite direction also occurs at the synaptic cleft: indeed, the process of trogocytosis, by which T cells can extract MHC:peptide complexes from APCs during endocytosis of engaged TCRs, has been shown to be important for sustained signaling at endosomes (88, 89). Together, these findings suggest that immune synapses are key facilitators of transcellular communication.

T_{FH} -derived cytokines can be secreted and signal to B cells in a contact-independent manner. However, it seems that cell–cell contact can potentially enhance the response. Indeed, secretion of cytokines across an immunological synapse can be highly effective due to the much higher concentrations that can be achieved within the synaptic space (90) are much more specific, because it is directed toward a B cell presenting cognate antigen. Variation in the amount of antigen presented can be detected in a highly sensitive manner, and the synapse can quickly change orientation to contact the cell presenting peptide:MHC complexes at the highest density. Together, these properties by which helper molecules get transmitted across an immune synapse are thought to enable T_{FH} cells to select those B cells expressing BCRs with the highest affinity for the immunizing antigen.

The observation made by Reinhardt and colleagues offers an example of this effective cytokine delivery. The isolation of T–B cell conjugates from the draining lymph node of immunized mice demonstrated that IgG1-producing B cells made contact with IL-4-producing T cells whereas IgG2a-producing B cells made contact with IFN- γ -producing T cells (91). IL-4-producing T cells were found conjugated to GC B cells expressing high levels of AID, with evidence of somatic hypermutation (91) demonstrating that T_{FH} -derived cytokines directly stimulate the production of different antibody isotypes in responding B cells sharing the same microenvironment. However, additional studies would be required to confirm these observations, perhaps using the LIPSTIC method, which allows direct measurement of dynamic cell–cell interactions both *in vitro* and *in vivo* (92).

Neurotransmitters in T–B Synapses

Neurotransmitters (NTs) are proteins used by the nervous system to communicate between neurons or other cells. This response typically occurs in response to changes in action potential when the neuron is activated. Substances acting as transmitters are stored in vesicles at synapses and are released by a process of exocytosis. Exocytosis in neurons occurs when depolarization of the neuron cell wall causes flux of calcium, binding of vesicles, and eventual externalization of vesicular content. Substances considered to be neurotransmitters are released into the synaptic cleft by exocytosis and/or directly from the cytoplasm. A neurotransmitter can be defined as a substance that is released by a neuron and that affects a specific target in a specific manner. A target can be either another neuron or an effector organ, such as muscle or gland. The concept of a transmitter is not precise, as neurotransmitters are protean,

structurally resembling other released agents in many regards. NTs act on targets that are close to the site of transmitter release, in distinction to hormones that are released in the bloodstream to act on distant targets (93). The interaction of neurotransmitters with receptors is typically transient, lasting from milliseconds to minutes. Despite the short timeframe of interaction, neurotransmitter action can result in long-term changes within target cells lasting hours or days.

Dopamine (DA) is a catecholamine mainly synthesized in the central nervous system where it acts as a neurotransmitter. The rate-limiting step in catecholamine synthesis is the conversion of tyrosine into L-DOPA by the enzyme tyrosine hydroxylase. Dopamine is then synthesized from L-DOPA by the enzyme DOPA decarboxylase. In the presence of other enzymes, dopamine can be further converted into noradrenaline and adrenaline.

In neurons, dopamine is packaged into vesicles after synthesis and can be released into the synaptic cleft upon the occurrence of a presynaptic action potential. Neuronal cells can also secrete dopamine into peripheral tissues. Furthermore, dopamine can also be synthesized within specific parenchymal tissue and endothelial cells (94, 95). Nevertheless, despite evidence of endothelial and other sources of peripheral dopamine production, the main contributor to plasma dopamine levels is production by sympathetic nerves.

An emerging role for dopamine in the immune system has recently been recognized. Dopamine can be produced by immune cells such as T cells and dendritic cells, and its release has various autocrine and paracrine effects (96–107).

Only recently, a role for dopamine in the GC reaction has been described (65). High dopamine amounts are found in T_{FH} cells compared to other T cell subsets analyzed. Dopamine is typically stored in dense core granules. Chromogranin B (CgB) is a marker of dense core secretory granules in the neuroendocrine system and is involved in the packaging of catecholamines, such as dopamine and noradrenaline. CgB⁺ granules are found in a small percentage of human T_{FH} cells. T_{FH} cells can synthesize dopamine upon cAMP induction and release it during T–B cell synapse formation. Once released, dopamine can bind to dopamine receptors expressed by human GC B cells and induce ICOSL translocation to the cell surface within minutes of stimulation. This effect appears to be mediated by dopamine receptor 1 (DRD1). Ligand of ICOS on human T_{FH} cells leads to fast translocation of CD40L to the center of the synapse. Furthermore, ICOS ligation also augmented the area of the T_{FH} –GC B cell synapse (65). This work adds to the growing evidence of the importance of ICOSL-mediated regulation of T cell help to GC B cells, required for productive GC reactions.

Cytotoxic Granules and Granzymes

Cytolytic granules are specialized secretory lysosomes containing a set of proteins, such as perforin and granzymes, involved in cell-mediated apoptosis (108, 109). Cytolytic granules can also be delivered through the immunological synapse. Specifically, this occurs through a secretory zone localized in the center of the synapse (110). Although cytotoxicity is a typical property of CD8⁺ T cells and natural killer cells, MHC class-II-restricted

cytotoxicity mediated by CD4⁺ T cells has also been described in both humans and mice (111–116). CD4⁺ CTL have been identified mostly during viral infections, suggesting that one of the main roles of CD4⁺ CTLs is antiviral immunity. CD4⁺ CTLs have also been identified during antitumor responses (117, 118) and chronic inflammatory responses (119–121).

Recent findings in mice described that the infecting or immunizing virus influences CD4⁺ CTL differentiation and that this differentiation program, once initiated, directly antagonizes T_{FH} differentiation. CD4 CTLs express high levels of Blimp1 and low levels of Bcl6, which is required for T_{FH} cell differentiation. Unlike the dependency of T_{FH} cells on BCL6 and TCF1, these transcription factors prevent CD4⁺ CTL induction, suggesting a dichotomous differentiation pathway between CD4⁺ T_{FH} and CTLs (122).

In human GCs a subset of T_{FH} cells expressing the surface marker CD57 (HNK-1/Leu-7) show cytotoxic activity (123). CD57 is generally upregulated in cells with cytotoxic activity (124). Further characterization of the nature and function of these granules will be required.

CONSEQUENCES OF T–B SYNAPTIC INTERACTIONS

In the nervous system, calcium fluxes are essential facilitators of synaptic neurotransmission. Action potentials open calcium channels in the presynaptic membrane causing the uptake of calcium ions (Ca²⁺). This calcium flux triggers the release of neurotransmitters from synaptic vesicles into the synaptic cleft. Calcium fluxes have been well studied in the context of T cell–APC synapse formation. It is only recently that calcium mobilization has been observed in the context of T_{FH}–GC B cell engagement.

Selection of high-affinity antibody-producing B cells is mediated by large but transient interactions between T_{FH} and GC B cells. It has been shown that in the presence of antigen, T cells reduce their speed and increase the duration and area of contact with high-affinity GC B cells. These interactions lead to an increase in T_{FH} intracellular calcium, which in turn increases the amount of helper cytokines IL-4 and IL-21 (125). Subsequent studies showed that mouse GC T cells help B cells in GCs *via* formation of entangled contacts, require extensive T and B cell surface interactions and rapid CD40L translocation to the surface

of T_{FH} cells. This translocation of preformed CD40L requires ICOS costimulation and calcium signaling (61). When ICOSL knockout B cells were engaged in interactions with T_{FH} cells, smaller calcium fluxes in the T cells were detected.

Ca²⁺ mobilization is also important in B cells after BCR engagement leading to different outcomes depending on the presence of additional signals. BCR engagement alone by antigen triggers a Ca²⁺ flux that causes downregulation of constitutive ICOSL surface expression on activated B cells (126, 127) and *in vitro* stimulated GC B cells (65). This downregulation is potentiated when BCRs are engaged together with IL4R activation, which acts through STAT6 to cause complete loss of ICOSL surface expression. By contrast, costimulation of B cells through CD40 signals (but not LPS or cytokines) after downregulation by antigen and/or IL-4 could restore expression of ICOSL. Restoration of ICOSL expression did not occur in B cells treated with anti-IgM. This was thought to be a consequence of high BCR crosslinking, since HEL stimulation, which does not crosslink BCRs, did not prevent ICOSL re-expression. Together these findings highlight that CD40–CD40L signaling and the nature of the antigen, control whether antigen-activated B cells are able to re-express ICOSL, which in turns leads to costimulation of the cognate T cell (126).

CONCLUDING REMARKS

The last decades of research in T–B cell interactions have revealed the importance of selective and focused delivery of important signals through the immunological synapse. Synaptic transmission of cytokines and neurotransmitters enables rapid regulation and translocation of molecules to the synaptic interface, required for effective T_{FH}-mediated B cell selection. Several molecules are known to be involved in the initiation of T–B immune synaptic transmission in GCs. Less is known about the signals required for the termination of T–B cell interactions, or about signals that distinguish T_{FH}–B vs T_{FR}–B cell interactions. We expect the coming years will uncover a much larger array of neurotransmitter-like molecules involved in T–B interactions in GCs.

AUTHOR CONTRIBUTIONS

IP and CV conceived, designed, and wrote the manuscript. CV revised the manuscript.

REFERENCES

1. Grakoui A, Bromley SK, Sumen C, Davis MM, Shaw AS, Allen PM, et al. The immunological synapse: a molecular machine controlling T cell activation. *Science* (1999) 285:221–7. doi:10.1126/science.285.5425.221
2. Paul WE, Seder RA. Lymphocyte responses and cytokines. *Cell* (1994) 76:241–51. doi:10.1016/0092-8674(94)90332-8
3. Dustin ML. The immunological synapse. *Cancer Immunol Res* (2014) 2:1023–33. doi:10.1158/2326-6066.CIR-14-0161
4. Bunnell SC, Hong DI, Kardon JR, Yamazaki T, McGlade CJ, Barr VA, et al. T cell receptor ligation induces the formation of dynamically regulated signaling assemblies. *J Cell Biol* (2002) 158:1263–75. doi:10.1083/jcb.200203043
5. Campi G, Varma R, Dustin ML. Actin and agonist MHC-peptide complex-dependent T cell receptor microclusters as scaffolds for signaling. *J Exp Med* (2005) 202:1031–6. doi:10.1084/jem.20051182
6. Hashimoto-Tane A, Yokosuka T, Sakata-Sogawa K, Sakuma M, Ishihara C, Tokunaga M, et al. Dynein-driven transport of T cell receptor microclusters regulates immune synapse formation and T cell activation. *Immunity* (2011) 34:919–31. doi:10.1016/j.immuni.2011.05.012
7. Yokosuka T, Sakata-Sogawa K, Kobayashi W, Hiroshima M, Hashimoto-Tane A, Tokunaga M, et al. Newly generated T cell receptor microclusters initiate and sustain T cell activation by recruitment of Zap70 and SLP-76. *Nat Immunol* (2005) 6:1253–62. doi:10.1038/ni1272
8. Monks CR, Freiberg BA, Kupfer H, Sciaky N, Kupfer A. Three-dimensional segregation of supramolecular activation clusters in T cells. *Nature* (1998) 395:82–6. doi:10.1038/25764
9. Davis SJ, van der Merwe PA. The kinetic-segregation model: TCR triggering and beyond. *Nat Immunol* (2006) 7:803–9. doi:10.1038/ni1369
10. Dustin ML, Bromley SK, Kan Z, Peterson DA, Unanue ER. Antigen receptor engagement delivers a stop signal to migrating T lymphocytes. *Proc Natl Acad Sci U S A* (1997) 94:3909–13. doi:10.1073/pnas.94.8.3909

11. Cemerski S, Das J, Locasale J, Arnold P, Giuriso E, Markiewicz MA, et al. The stimulatory potency of T cell antigens is influenced by the formation of the immunological synapse. *Immunity* (2007) 26:345–55. doi:10.1016/j.immuni.2007.01.013
12. Kaizuka Y, Douglass AD, Vardhana S, Dustin ML, Vale RD. The coreceptor CD2 uses plasma membrane microdomains to transduce signals in T cells. *J Cell Biol* (2009) 185:521–34. doi:10.1083/jcb.200809136
13. Bromley SK, Iaboni A, Davis SJ, Whitty A, Green JM, Shaw AS, et al. The immunological synapse and CD28-CD80 interactions. *Nat Immunol* (2001) 2:1159–66. doi:10.1038/ni737
14. Lafferty KJ, Cunningham AJ. A new analysis of allogeneic interactions. *Aust J Exp Biol Med Sci* (1975) 53:27–42. doi:10.1038/icb.1975.3
15. Martin PJ, Ledbetter JA, Morishita Y, June CH, Beatty PG, Hansen JA. A 44 kilodalton cell surface homodimer regulates interleukin 2 production by activated human T lymphocytes. *J Immunol* (1986) 136:3282–7.
16. Kong KF, Yokosuka T, Canonigo-Balancio AJ, Isakov N, Saito T, Altman A. A motif in the V3 domain of the kinase PKC- θ determines its localization in the immunological synapse and functions in T cells via association with CD28. *Nat Immunol* (2011) 12:1105–12. doi:10.1038/ni.2120
17. Tseng SY, Waite JC, Liu M, Vardhana S, Dustin ML. T cell-dendritic cell immunological synapses contain TCR-dependent CD28-CD80 clusters that recruit protein kinase C θ . *J Immunol* (2008) 181:4852–63. doi:10.4049/jimmunol.181.7.4852
18. Inaba K, Witmer-Pack M, Inaba M, Hathcock KS, Sakuta H, Azuma M, et al. The tissue distribution of the B7-2 costimulator in mice: abundant expression on dendritic cells in situ and during maturation in vitro. *J Exp Med* (1994) 180:1849–60. doi:10.1084/jem.180.5.1849
19. Medzhitov R, Preston-Hurlburt P, Janeway CA Jr. A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. *Nature* (1997) 388:394–7. doi:10.1038/41131
20. Fos C, Salles A, Lang V, Carrette F, Audebert S, Pastor S, et al. ICOS ligation recruits the p50 α PI3K regulatory subunit to the immunological synapse. *J Immunol* (2008) 181:1969–77. doi:10.4049/jimmunol.181.3.1969
21. Batista FD, Iber D, Neuberger MS. B cells acquire antigen from target cells after synapse formation. *Nature* (2001) 411:489–94. doi:10.1038/35078099
22. Fleire SJ, Goldman JP, Carrasco YR, Weber M, Bray D, Batista FD. B cell ligand discrimination through a spreading and contraction response. *Science* (2006) 312:738–41. doi:10.1126/science.1123940
23. Natkanski E, Lee WY, Mistry B, Casal A, Molloy JE, Tolar P. B cells use mechanical energy to discriminate antigen affinities. *Science* (2013) 340:1587–90. doi:10.1126/science.1237572
24. Nowosad CR, Spillane KM, Tolar P. Germinal center B cells recognize antigen through a specialized immune synapse architecture. *Nat Immunol* (2016) 17:870–7. doi:10.1038/ni.3458
25. Allen CD, Okada T, Tang HL, Cyster JG. Imaging of germinal center selection events during affinity maturation. *Science* (2007) 315:528–31. doi:10.1126/science.1136736
26. Yu D, Cook MC, Shin DM, Silva DG, Marshall J, Toellner KM, et al. Axon growth and guidance genes identify T-dependent germinal centre B cells. *Immunol Cell Biol* (2008) 86:3–14. doi:10.1038/sj.icb.7100123
27. Spillane KM, Tolar P. B cell antigen extraction is regulated by physical properties of antigen-presenting cells. *J Cell Biol* (2017) 216:217–30. doi:10.1083/jcb.201607064
28. Koopman G, Keehnen RM, Lindhout E, Newman W, Shimizu Y, van Seventer GA, et al. Adhesion through the LFA-1 (CD11a/CD18)-ICAM-1 (CD54) and the VLA-4 (CD49d)-VCAM-1 (CD106) pathways prevents apoptosis of germinal center B cells. *J Immunol* (1994) 152:3760–7.
29. Victoratos P, Lagnel J, Tzima S, Alimzhanov MB, Rajewsky K, Pasparakis M, et al. FDC-specific functions of p55TNFR and IKK2 in the development of FDC networks and of antibody responses. *Immunity* (2006) 24:65–77. doi:10.1016/j.immuni.2005.11.013
30. El Shikh ME, El Sayed R, Szakal AK, Tew JG. Follicular dendritic cell (FDC)-Fc γ RIIB engagement via immune complexes induces the activated FDC phenotype associated with secondary follicle development. *Eur J Immunol* (2006) 36:2715–24. doi:10.1002/eji.200636122
31. Lindhout E, Mevissen ML, Kwekkeboom J, Tager JM, de Groot C. Direct evidence that human follicular dendritic cells (FDC) rescue germinal centre B cells from death by apoptosis. *Clin Exp Immunol* (1993) 91:330–6. doi:10.1111/j.1365-2249.1993.tb05904.x
32. Wang X, Cho B, Suzuki K, Xu Y, Green JA, An J, et al. Follicular dendritic cells help establish follicle identity and promote B cell retention in germinal centers. *J Exp Med* (2011) 208:2497–510. doi:10.1084/jem.20111449
33. Randall KL, Lambe T, Johnson AL, Treanor B, Kucharska E, Domschensch H, et al. Dock8 mutations cripple B cell immunological synapses, germinal centers and long-lived antibody production. *Nat Immunol* (2009) 10:1283–91. doi:10.1038/ni.1820
34. Wang X, Rodda LB, Bannard O, Cyster JG. Integrin-mediated interactions between B cells and follicular dendritic cells influence germinal center B cell fitness. *J Immunol* (2014) 192:4601–9. doi:10.4049/jimmunol.1400090
35. Rahman ZS, Rao SP, Kalled SL, Manser T. Normal induction but attenuated progression of germinal center responses in BAFF and BAFF-R signaling-deficient mice. *J Exp Med* (2003) 198:1157–69. doi:10.1084/jem.20030495
36. Shulga-Morskaya S, Dobles M, Walsh ME, Ng LG, MacKay F, Rao SP, et al. B cell-activating factor belonging to the TNF family acts through separate receptors to support B cell survival and T cell-independent antibody formation. *J Immunol* (2004) 173:2331–41. doi:10.4049/jimmunol.173.4.2331
37. Vora KA, Wang LC, Rao SP, Liu ZY, Majeau GR, Cutler AH, et al. Cutting edge: germinal centers formed in the absence of B cell-activating factor belonging to the TNF family exhibit impaired maturation and function. *J Immunol* (2003) 171:547–51. doi:10.4049/jimmunol.171.2.547
38. von Bulow GU, van Deursen JM, Bram RJ. Regulation of the T-independent humoral response by TACI. *Immunity* (2001) 14:573–82. doi:10.1016/S1074-7613(01)00130-3
39. Xu S, Lam KP. B-cell maturation protein, which binds the tumor necrosis factor family members BAFF and APRIL, is dispensable for humoral immune responses. *Mol Cell Biol* (2001) 21:4067–74. doi:10.1128/MCB.21.12.4067-4074.2001
40. Gardam S, Brink R. Non-canonical NF- κ B signaling initiated by BAFF influences B cell biology at multiple junctures. *Front Immunol* (2014) 4:509. doi:10.3389/fimmu.2013.00509
41. Husson H, Lugli SM, Ghia P, Cardoso A, Roth A, Brohmi K, et al. Functional effects of TNF and lymphotoxin α 1 β 2 on FDC-like cells. *Cell Immunol* (2000) 203:134–43. doi:10.1006/cimm.2000.1688
42. Li L, Zhang X, Kovacic S, Long AJ, Bourque K, Wood CR, et al. Identification of a human follicular dendritic cell molecule that stimulates germinal center B cell growth. *J Exp Med* (2000) 191:1077–84. doi:10.1084/jem.191.6.1077
43. Zhang X, Li L, Jung J, Xiang S, Hollmann C, Choi YS. The distinct roles of T cell-derived cytokines and a novel follicular dendritic cell-signaling molecule 8D6 in germinal center-B cell differentiation. *J Immunol* (2001) 167:49–56. doi:10.4049/jimmunol.167.1.49
44. Gil M, Park SJ, Chung YS, Park CS. Interleukin-15 enhances proliferation and chemokine secretion of human follicular dendritic cells. *Immunology* (2010) 130:536–44. doi:10.1111/j.1365-2567.2010.03252.x
45. Park CS, Yoon SO, Armitage RJ, Choi YS. Follicular dendritic cells produce IL-15 that enhances germinal center B cell proliferation in membrane-bound form. *J Immunol* (2004) 173:6676–83. doi:10.4049/jimmunol.173.11.6676
46. Kopf M, Herren S, Wiles MV, Pepys MB, Kosco-Vilbois MH. Interleukin 6 influences germinal center development and antibody production via a contribution of C3 complement component. *J Exp Med* (1998) 188:1895–906. doi:10.1084/jem.188.10.1895
47. Harker JA, Lewis GM, Mack L, Zuniga EI. Late interleukin-6 escalates T follicular helper cell responses and controls a chronic viral infection. *Science* (2011) 334:825–9. doi:10.1126/science.1208421
48. Nurieva RI, Chung Y, Martinez GJ, Yang XO, Tanaka S, Matskevitch TD, et al. Bcl6 mediates the development of T follicular helper cells. *Science* (2009) 325:1001–5. doi:10.1126/science.1176676
49. Boles KS, Vermi W, Facchetti F, Fuchs A, Wilson TJ, Diacovo TG, et al. A novel molecular interaction for the adhesion of follicular CD4 T cells to follicular DC. *Eur J Immunol* (2009) 39:695–703. doi:10.1002/eji.200839116
50. Locci M, Havenar-Daughton C, Landais E, Wu J, Kroenke MA, Arlehamn CL, et al. Human circulating PD-1+CXCR3-CXCR5+ memory T_H cells are highly functional and correlate with broadly neutralizing HIV antibody responses. *Immunity* (2013) 39:758–69. doi:10.1016/j.immuni.2013.08.031
51. Godefroy E, Zhong H, Pham P, Friedman D, Yazdanbakhsh K. TIGIT-positive circulating follicular helper T cells display robust B-cell help functions: potential role in sickle cell alloimmunization. *Haematologica* (2015) 100:1415–25. doi:10.3324/haematol.2015.132738

52. Yu X, Harden K, Gonzalez LC, Francesco M, Chiang E, Irving B, et al. The surface protein TIGIT suppresses T cell activation by promoting the generation of mature immunoregulatory dendritic cells. *Nat Immunol* (2009) 10:48–57. doi:10.1038/ni.1674
53. Evans R, Patzak I, Svensson L, De Filippo K, Jones K, McDowall A, et al. Integrins in immunity. *J Cell Sci* (2009) 122:215–25. doi:10.1242/jcs.019117
54. Brunmark A, O'Rourke AM. Augmentation of mature CD4+ T cell responses to isolated antigenic class II proteins by fibronectin and intercellular adhesion molecule-1. *J Immunol* (1997) 159:1676–85.
55. Wulfig C, Sjaastad MD, Davis MM. Visualizing the dynamics of T cell activation: intracellular adhesion molecule 1 migrates rapidly to the T cell/B cell interface and acts to sustain calcium levels. *Proc Natl Acad Sci U S A* (1998) 95:6302–7. doi:10.1073/pnas.95.11.6302
56. Meli AP, Fontes G, Avery DT, Leddon SA, Tam M, Elliot M, et al. The integrin LFA-1 controls T follicular helper cell generation and maintenance. *Immunity* (2016) 45:831–46. doi:10.1016/j.immuni.2016.09.018
57. Zaretsky I, Atrakchi O, Mazor RD, Stoler-Barak L, Biram A, Feigelson SW, et al. ICAMs support B cell interactions with T follicular helper cells and promote clonal selection. *J Exp Med* (2017) 214:3435–48. doi:10.1084/jem.20171129
58. Liu Y, Blanchfield L, Ma VP, Andargachew R, Galior K, Liu Z, et al. DNA-based nanoparticle tension sensors reveal that T-cell receptors transmit defined pN forces to their antigens for enhanced fidelity. *Proc Natl Acad Sci U S A* (2016) 113:5610–5. doi:10.1073/pnas.1600163113
59. Han S, Hathcock K, Zheng B, Kepler TB, Hodes R, Kelsoe G. Cellular interaction in germinal centers. Roles of CD40 ligand and B7-2 in established germinal centers. *J Immunol* (1995) 155:556–67.
60. Liu YJ, Joshua DE, Williams GT, Smith CA, Gordon J, MacLennan IC. Mechanism of antigen-driven selection in germinal centres. *Nature* (1989) 342:929–31. doi:10.1038/342929a0
61. Liu D, Xu H, Shih C, Wan Z, Ma X, Ma W, et al. T-B-cell entanglement and ICOSL-driven feed-forward regulation of germinal centre reaction. *Nature* (2015) 517:214–8. doi:10.1038/nature13803
62. Casamayor-Palleja M, Khan M, MacLennan IC. A subset of CD4+ memory T cells contains preformed CD40 ligand that is rapidly but transiently expressed on their surface after activation through the T cell receptor complex. *J Exp Med* (1995) 181:1293–301. doi:10.1084/jem.181.4.1293
63. Gardell JL, Parker DC. Despite disorganized synapse structure, Th2 cells maintain directional delivery of CD40L to antigen-presenting B cells. *PLoS One* (2017) 12:e0186573. doi:10.1371/journal.pone.0186573
64. Thauland TJ, Koguchi Y, Wetzel SA, Dustin ML, Parker DC. Th1 and Th2 cells form morphologically distinct immunological synapses. *J Immunol* (2008) 181:393–9. doi:10.4049/jimmunol.181.1.393
65. Papa I, Saliba D, Ponzoni M, Bustamante S, Canete PF, Gonzalez-Figueroa P, et al. TFH-derived dopamine accelerates productive synapses in germinal centres. *Nature* (2017) 547:318–23. doi:10.1038/nature23013
66. Cannons JL, Qi H, Lu KT, Dutta M, Gomez-Rodriguez J, Cheng J, et al. Optimal germinal center responses require a multistage T cell:B cell adhesion process involving integrins, SLAM-associated protein, and CD84. *Immunity* (2010) 32:253–65. doi:10.1016/j.immuni.2010.01.010
67. Cannons JL, Tangye SG, Schwartzberg PL. SLAM family receptors and SAP adaptors in immunity. *Annu Rev Immunol* (2011) 29:665–705. doi:10.1146/annurev-immunol-030409-101302
68. Chtanova T, Tangye SG, Newton R, Frank N, Hodge MR, Rolph MS, et al. T follicular helper cells express a distinctive transcriptional profile, reflecting their role as non-Th1/Th2 effector cells that provide help for B cells. *J Immunol* (2004) 173:68–78. doi:10.4049/jimmunol.173.1.68
69. Ma CS, Suryani S, Avery DT, Chan A, Nanan R, Santner-Nanan B, et al. Early commitment of naive human CD4(+) T cells to the T follicular helper (TFH) cell lineage is induced by IL-12. *Immunol Cell Biol* (2009) 87:590–600. doi:10.1038/icb.2009.64
70. Qi H, Cannons JL, Klauschen F, Schwartzberg PL, Germain RN. SAP-controlled T-B cell interactions underlie germinal centre formation. *Nature* (2008) 455:764–9. doi:10.1038/nature07345
71. Schwartzberg PL, Mueller KL, Qi H, Cannons JL. SLAM receptors and SAP influence lymphocyte interactions, development and function. *Nat Rev Immunol* (2009) 9:39–46. doi:10.1038/nri2456
72. Chevalier N, Macia L, Tan JK, Mason LJ, Robert R, Thorburn AN, et al. The role of follicular helper T cell molecules and environmental influences in autoantibody production and progression to inflammatory arthritis in mice. *Arthritis Rheumatol* (2016) 68:1026–38. doi:10.1002/art.39481
73. Kim J, Kim YM, Jeoung DI, Choe J. Human follicular dendritic cells promote the APC capability of B cells by enhancing CD86 expression levels. *Cell Immunol* (2012) 273:109–14. doi:10.1016/j.cellimm.2012.01.003
74. Attridge K, Kenefeck R, Wardzinski L, Qureshi OS, Wang CJ, Manzotti C, et al. IL-21 promotes CD4 T cell responses by phosphatidylinositol 3-kinase-dependent upregulation of CD86 on B cells. *J Immunol* (2014) 192:2195–201. doi:10.4049/jimmunol.1302082
75. Wang CJ, Heuts F, Ovcinnikovs V, Wardzinski L, Bowers C, Schmidt EM, et al. CTLA-4 controls follicular helper T-cell differentiation by regulating the strength of CD28 engagement. *Proc Natl Acad Sci U S A* (2015) 112:524–9. doi:10.1073/pnas.1414576112
76. Pasterkamp RJ. Getting neural circuits into shape with semaphorins. *Nat Rev Neurosci* (2012) 13:605–18. doi:10.1038/nrn3302
77. Tamagnone L, Artigiani S, Chen H, He Z, Ming GI, Song H, et al. Plexins are a large family of receptors for transmembrane, secreted, and GPI-anchored semaphorins in vertebrates. *Cell* (1999) 99:71–80. doi:10.1016/S0092-8674(00)80063-X
78. Yan H, Wu L, Shih C, Hou S, Shi J, Mao T, et al. Plexin B2 and semaphorin 4C guide T cell recruitment and function in the germinal center. *Cell Rep* (2017) 19:995–1007. doi:10.1016/j.celrep.2017.04.022
79. Lu P, Shih C, Qi H. Ephrin B1-mediated repulsion and signaling control germinal center T cell territoriality and function. *Science* (2017) 356(6339):eaai9264. doi:10.1126/science.aai9264
80. Laidlaw BJ, Schmidt TH, Green JA, Allen CD, Okada T, Cyster JG. The Eph-related tyrosine kinase ligand Ephrin-B1 marks germinal center and memory precursor B cells. *J Exp Med* (2017) 214:639–49. doi:10.1084/jem.20161461
81. Ostroumova OS, Schagina LV, Mosevitsky MI, Zakharov VV. Ion channel activity of brain abundant protein BASP1 in planar lipid bilayers. *FEBS J* (2011) 278:461–9. doi:10.1111/j.1742-4658.2010.07967.x
82. Frey D, Laux T, Xu L, Schneider C, Caroni P. Shared and unique roles of CAP23 and GAP43 in actin regulation, neurite outgrowth, and anatomical plasticity. *J Cell Biol* (2000) 149:1443–54. doi:10.1083/jcb.149.7.1443
83. Geiger B, Rosen D, Berke G. Spatial relationships of microtubule-organizing centers and the contact area of cytotoxic T lymphocytes and target cells. *J Cell Biol* (1982) 95:137–43. doi:10.1083/jcb.95.1.137
84. Kupfer A, Swain SL, Janeway CA Jr, Singer SJ. The specific direct interaction of helper T cells and antigen-presenting B cells. *Proc Natl Acad Sci U S A* (1986) 83:6080–3. doi:10.1073/pnas.83.16.6080
85. Poo WJ, Conrad L, Janeway CA Jr. Receptor-directed focusing of lymphokine release by helper T cells. *Nature* (1988) 332:378–80. doi:10.1038/332378a0
86. Choudhuri K, Llodra J, Roth EW, Tsai J, Gordo S, Wucherpfennig KW, et al. Polarized release of T-cell-receptor-enriched microvesicles at the immunological synapse. *Nature* (2014) 507:118–23. doi:10.1038/nature12951
87. Mittelbrunn M, Gutierrez-Vazquez C, Villarroya-Beltri C, Gonzalez S, Sanchez-Cabo F, Gonzalez MA, et al. Unidirectional transfer of microRNA-loaded exosomes from T cells to antigen-presenting cells. *Nat Commun* (2011) 2:282. doi:10.1038/ncomms1285
88. Huang JF, Yang Y, Sepulveda H, Shi W, Hwang I, Peterson PA, et al. TCR-mediated internalization of peptide-MHC complexes acquired by T cells. *Science* (1999) 286:952–4. doi:10.1126/science.286.5441.952
89. Wetzel SA, McKeithan TW, Parker DC. Peptide-specific intercellular transfer of MHC class II to CD4+ T cells directly from the immunological synapse upon cellular dissociation. *J Immunol* (2005) 174:80–9. doi:10.4049/jimmunol.174.1.80
90. Huse M, Lillemeier BF, Kuhns MS, Chen DS, Davis MM. T cells use two directionally distinct pathways for cytokine secretion. *Nat Immunol* (2006) 7:247–55. doi:10.1038/ni1304
91. Reinhardt RL, Liang HE, Locksley RM. Cytokine-secreting follicular T cells shape the antibody repertoire. *Nat Immunol* (2009) 10:385–93. doi:10.1038/ni.1715
92. Pasqual G, Chudnovskiy A, Tas JMJ, Agudelo M, Schweitzer LD, Cui A, et al. Monitoring T cell-dendritic cell interactions in vivo by intercellular enzymatic labelling. *Nature* (2018) 553:496–500. doi:10.1038/nature25442
93. Kandel ER, Schwartz JH, Jessell TM, Siegelbaum SA, Hudspeth AJ. *Principles of Neural Science*. 5th ed. US: McGraw-Hill (2013).

94. Rubi B, Maechler P. Minireview: new roles for peripheral dopamine on metabolic control and tumor growth: let's seek the balance. *Endocrinology* (2010) 151:5570–81. doi:10.1210/en.2010-0745
95. Sorriento D, Santulli G, Del Giudice C, Anastasio A, Trimarco B, Iaccarino G. Endothelial cells are able to synthesize and release catecholamines both in vitro and in vivo. *Hypertension* (2012) 60:129–36. doi:10.1161/HYPERTENSIONAHA.111.189605
96. Bergquist J, Josefsson E, Tarkowski A, Ekman R, Ewing A. Measurements of catecholamine-mediated apoptosis of immunocompetent cells by capillary electrophoresis. *Electrophoresis* (1997) 18:1760–6. doi:10.1002/elps.1150181009
97. Bergquist J, Silberring J. Identification of catecholamines in the immune system by electrospray ionization mass spectrometry. *Rapid Commun Mass Spectrom* (1998) 12:683–8. doi:10.1002/(SICI)1097-0231(19980615)12:11<683::AID-RCM218>3.0.CO;2-N
98. Bergquist J, Tarkowski A, Ekman R, Ewing A. Discovery of endogenous catecholamines in lymphocytes and evidence for catecholamine regulation of lymphocyte function via an autocrine loop. *Proc Natl Acad Sci U S A* (1994) 91:12912–6. doi:10.1073/pnas.91.26.12912
99. Cosentino M, Fietta AM, Ferrari M, Rasini E, Bombelli R, Carcano E, et al. Human CD4+CD25+ regulatory T cells selectively express tyrosine hydroxylase and contain endogenous catecholamines subserving an autocrine/paracrine inhibitory functional loop. *Blood* (2007) 109:632–42. doi:10.1182/blood-2006-01-028423
100. Ferrari M, Cosentino M, Marino F, Bombelli R, Rasini E, Lecchini S, et al. Dopaminergic D1-like receptor-dependent inhibition of tyrosine hydroxylase mRNA expression and catecholamine production in human lymphocytes. *Biochem Pharmacol* (2004) 67:865–73. doi:10.1016/j.bcp.2003.10.004
101. Flierl MA, Rittirsch D, Nadeau BA, Chen AJ, Sarma JV, Zetoune FS, et al. Phagocyte-derived catecholamines enhance acute inflammatory injury. *Nature* (2007) 449:721–5. doi:10.1038/nature06185
102. Flierl MA, Rittirsch D, Nadeau BA, Sarma JV, Day DE, Lentsch AB, et al. Upregulation of phagocyte-derived catecholamines augments the acute inflammatory response. *PLoS One* (2009) 4:e4414. doi:10.1371/journal.pone.0004414
103. Josefsson E, Bergquist J, Ekman R, Tarkowski A. Catecholamines are synthesized by mouse lymphocytes and regulate function of these cells by induction of apoptosis. *Immunology* (1996) 88:140–6. doi:10.1046/j.1365-2567.1996.d01-653.x
104. Musso NR, Brenci S, Indiveri F, Lotti G. L-tyrosine and nicotine induce synthesis of L-Dopa and norepinephrine in human lymphocytes. *J Neuroimmunol* (1997) 74:117–20. doi:10.1016/S0165-5728(96)00212-3
105. Musso NR, Brenci S, Setti M, Indiveri F, Lotti G. Catecholamine content and in vitro catecholamine synthesis in peripheral human lymphocytes. *J Clin Endocrinol Metab* (1996) 81:3553–7. doi:10.1210/jcem.81.10.8855800
106. Nakano K, Higashi T, Takagi R, Hashimoto K, Tanaka Y, Matsushita S. Dopamine released by dendritic cells polarizes Th2 differentiation. *Int Immunol* (2009) 21:645–54. doi:10.1093/intimm/dxp033
107. Tsao CW, Lin YS, Cheng JT. Inhibition of immune cell proliferation with haloperidol and relationship of tyrosine hydroxylase expression to immune cell growth. *Life Sci* (1998) 62:335–44. doi:10.1016/S0024-3205(98)00170-2
108. Griffiths GM, Isaaz S, Granzymes A and B are targeted to the lytic granules of lymphocytes by the mannose-6-phosphate receptor. *J Cell Biol* (1993) 120:885–96. doi:10.1083/jcb.120.4.885
109. Shresta S, Pham CT, Thomas DA, Graubert TA, Ley TJ. How do cytotoxic lymphocytes kill their targets? *Curr Opin Immunol* (1998) 10:581–7. doi:10.1016/S0952-7915(98)80227-6
110. Stinchcombe JC, Griffiths GM. Secretory mechanisms in cell-mediated cytotoxicity. *Annu Rev Cell Dev Biol* (2007) 23:495–517. doi:10.1146/annurev.cellbio.23.090506.123521
111. Brown DM. Cytolytic CD4 cells: direct mediators in infectious disease and malignancy. *Cell Immunol* (2010) 262:89–95. doi:10.1016/j.cellimm.2010.02.008
112. Brown DM, Lampe AT, Workman AM. The differentiation and protective function of cytolytic CD4 T cells in influenza infection. *Front Immunol* (2016) 7:93. doi:10.3389/fimmu.2016.00093
113. Cheroutre H, Husain MM. CD4 CTL: living up to the challenge. *Semin Immunol* (2013) 25:273–81. doi:10.1016/j.smim.2013.10.022
114. Soghoian DZ, Streeck H. Cytolytic CD4(+) T cells in viral immunity. *Expert Rev Vaccines* (2010) 9:1453–63. doi:10.1586/erv.10.132
115. Swain SL, McKinstry KK, Strutt TM. Expanding roles for CD4(+) T cells in immunity to viruses. *Nat Rev Immunol* (2012) 12:136–48. doi:10.1038/nri3152
116. Takeuchi A, Saito T. CD4 CTL, a cytotoxic subset of CD4(+) T cells, their differentiation and function. *Front Immunol* (2017) 8:194. doi:10.3389/fimmu.2017.00194
117. Quezada SA, Simpson TR, Peggs KS, Merghoub T, Vider J, Fan X, et al. Tumor-reactive CD4(+) T cells develop cytotoxic activity and eradicate large established melanoma after transfer into lymphopenic hosts. *J Exp Med* (2010) 207:637–50. doi:10.1084/jem.20091918
118. Xie Y, Akpınarlı A, Maris C, Hipkiss EL, Lane M, Kwon EK, et al. Naive tumor-specific CD4(+) T cells differentiated in vivo eradicate established melanoma. *J Exp Med* (2010) 207:651–67. doi:10.1084/jem.20091921
119. Peeters LM, Vanheusden M, Somers V, Van Wijmeersch B, Stinissen P, Broux B, et al. Cytotoxic CD4+ T cells drive multiple sclerosis progression. *Front Immunol* (2017) 8:1160. doi:10.3389/fimmu.2017.01160
120. Thewissen M, Somers V, Hellings N, Fraussen J, Damoiseaux J, Stinissen P. CD4+CD28null T cells in autoimmune disease: pathogenic features and decreased susceptibility to immunoregulation. *J Immunol* (2007) 179:6514–23. doi:10.4049/jimmunol.179.10.6514
121. van de Berg PJ, van Leeuwen EM, ten Berge IJ, van Lier R. Cytotoxic human CD4(+) T cells. *Curr Opin Immunol* (2008) 20:339–43. doi:10.1016/j.coi.2008.03.007
122. Donnarumma T, Young GR, Merkenschlager J, Eksmond U, Bongard N, Nutt SL, et al. Opposing development of cytotoxic and follicular helper CD4 T cells controlled by the TCF-1-Bcl6 nexus. *Cell Rep* (2016) 17:1571–83. doi:10.1016/j.celrep.2016.10.013
123. Alshekaili J, Chand R, Lee CE, Corley S, Kwong K, Papa I, et al. STAT3 regulates cytotoxicity of human CD57+ CD4+ T cells in blood and lymphoid follicles. *Sci Rep* (2018) 8:3529. doi:10.1038/s41598-018-21389-8
124. Nielsen CM, White MJ, Goodier MR, Riley EM. Functional significance of CD57 expression on human NK cells and relevance to disease. *Front Immunol* (2013) 4:422. doi:10.3389/fimmu.2013.00422
125. Shulman Z, Gitlin AD, Weinstein JS, Lainez B, Esplugues E, Flavell RA, et al. Dynamic signaling by T follicular helper cells during germinal center B cell selection. *Science* (2014) 345:1058–62. doi:10.1126/science.1257861
126. Liang L, Porter EM, Sha WC. Constitutive expression of the B7h ligand for inducible costimulator on naive B cells is extinguished after activation by distinct B cell receptor and interleukin 4 receptor-mediated pathways and can be rescued by CD40 signaling. *J Exp Med* (2002) 196:97–108. doi:10.1084/jem.20020298
127. Sacquin A, Gador M, Fazilleau N. The strength of BCR signaling shapes terminal development of follicular helper T cells in mice. *Eur J Immunol* (2017) 47:1295–304. doi:10.1002/eji.201746952

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2018 Papa and Vinuesa. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



Insights Into the Molecular Mechanisms of T Follicular Helper-Mediated Immunity and Pathology

Lei Qin^{1,2†}, Tayab C. Waseem^{3†}, Anupama Sahoo^{1†}, Shayahati Bieerkehazhi¹, Hong Zhou², Elena V. Galkina³ and Roza Nurieva^{1*}

¹ Department of Immunology, University of Texas MD Anderson Cancer Center, Houston, TX, United States, ² School of Life Science and Technology, University of Electronic Science and Technology of China, Chengdu, China, ³ Department of Microbiology and Molecular Cell Biology, Eastern Virginia Medical School, Norfolk, VA, United States

OPEN ACCESS

Edited by:

Maria Pia Cicalese,
San Raffaele Scientific Institute
(IRCCS), Italy

Reviewed by:

Jinfang Zhu,
National Institute of Allergy and
Infectious Diseases (NIAID),
United States
John R. Lukens,
University of Virginia, United States

*Correspondence:

Roza Nurieva
nurieva@mdanderson.org

[†]These authors have contributed
equally to this work.

Specialty section:

This article was submitted to
T Cell Biology,
a section of the journal
Frontiers in Immunology

Received: 15 June 2018

Accepted: 31 July 2018

Published: 15 August 2018

Citation:

Qin L, Waseem TC, Sahoo A,
Bieerkehazhi S, Zhou H, Galkina EV
and Nurieva R (2018) Insights Into the
Molecular Mechanisms of T Follicular
Helper-Mediated Immunity and
Pathology.
Front. Immunol. 9:1884.
doi: 10.3389/fimmu.2018.01884

T follicular helper (Tfh) cells play key role in providing help to B cells during germinal center (GC) reactions. Generation of protective antibodies against various infections is an important aspect of Tfh-mediated immune responses and the dysregulation of Tfh cell responses has been implicated in various autoimmune disorders, inflammation, and malignancy. Thus, their differentiation and maintenance must be closely regulated to ensure appropriate help to B cells. The generation and function of Tfh cells is regulated by multiple checkpoints including their early priming stage in T zones and throughout the effector stage of differentiation in GCs. Signaling pathways activated downstream of cytokine and costimulatory receptors as well as consequent activation of subset-specific transcriptional factors are essential steps for Tfh cell generation. Thus, understanding the mechanisms underlying Tfh cell-mediated immunity and pathology will bring into spotlight potential targets for novel therapies. In this review, we discuss the recent findings related to the molecular mechanisms of Tfh cell differentiation and their role in normal immune responses and antibody-mediated diseases.

Keywords: T follicular helper cells, germinal center, transcription, autoimmunity, cancer

INTRODUCTION

Germinal centers (GCs) are secondary lymphoid structures within B cell follicles where B cells go through affinity maturation and class-switch recombination to generate high-affinity antibodies (1, 2). GC reactions play a critical role in the invasion of pathogens, while abnormal GC reactions are implicated in systemic autoimmune diseases, chronic inflammation, allergic responses, and B cell malignancies. The GC reaction is initiated and amplified by GC B cell and CD4⁺ T cell interactions followed by T follicular helper (Tfh) cell help to B cells which leads to the generation of long-lived serological memory (2, 3). Exaggerated expansion of Tfh cells results in excessive GC reactions, self-reactive B cell proliferation, and increased long-lived plasma cell differentiation, as well as an overproduction of high-affinity pathogenic autoantibodies (4). Understanding the development and function of Tfh cells is important for generating new vaccine strategies against pathogens as well as targeted approaches to abrogate the inappropriate activity of these cells in patients with various autoimmune diseases.

T follicular helper cell development occurs in a stepwise process (1, 2). Contact of naïve CD4⁺ T cells with antigen-presenting dendritic cells (DCs) within T cell follicles is the first step of commitment toward Tfh cell differentiation (**Figure 1**). This step of Th cell development, named as pre-Tfh is reflected by upregulation of CXC chemokine receptor 5 (CXCR5) expression as well as key genes in the Tfh pathway such as B-cell lymphoma 6 protein (Bcl6), Achaete-scute homolog 2 (Ascl2), ICOS, programmed cell death-1 (PD-1), and Batf, and the downregulation of CC chemokine receptor 7 (CCR7) expression. These changes guide the pre-Tfh cells to the T/B cell border where proper signals received from B cells trigger a further increase in the Tfh-associated gene expression pattern (Bcl6, PD-1, ICOS, and CXCR5), commitment to the functional GC Tfh cell program and subsequent GC formation. While in mice, IL-6, IL-21, and Bcl6 are essential for Tfh formation, in humans, Tfh generation relies on TGF- β , IL-12, IL-23, and Activin A signaling (5–9). In addition, Tfh cell differentiation is negatively controlled by cytokines (IL-2 and IL-7) costimulatory molecules [cytotoxic T lymphocyte antigen 4 (CTLA4) and PD-1], and transcriptional factors [signal transducers and activators of transcription (STAT)5, Blimp-1, FOXO1, Foxp1, and Krüppel-like factor 2 (Klf2)] (10–16).

In this review, we have discussed positive and negative regulation mechanisms of mouse and human Tfh differentiation including costimulation and cytokine driven signaling pathways and subsequent activation of downstream transcriptional factors. We also elaborate on the cellular

requirement of other follicular T cells within GCs for Tfh cell development and GC formation. Moreover, we will review the current advances in Tfh cell biology in various disease settings.

Tfh CELLS IN MICE AND HUMANS

Several seminal discoveries made in humans and mice in the early 2000s led to identification of B follicular helper T cells that are indispensable for GC formation and B cell function (17, 18). These cells express high levels of CXCR5 and low CCR7 in both humans and mice (17–20). CXCR5 expression is indispensable for T cell migration from T zones toward CXCL13-rich B-cell follicles, where they interact with B cells for further maturation and then provide help for the generation of high-affinity antibodies and long-lived plasma cells (21–24). Thus, based on their localization and function, CXCR5⁺ CD4⁺ T cells were designated as Tfh cells.

Profiling of cytokine and gene expression patterns provided the evidence that mouse and human Tfh cells are distinct from Th1 and Th2 subsets and help B cells by delivering activating signals with CD40L and the cytokine IL-21 (3, 6, 18, 25). Moreover, these mouse and human gene-profiling experiments helped to identify key molecules including Bcl6, Ascl2, IL-21, PD-1, and ICOS which play a substantial role in Tfh cell development, migration, homeostasis, and function in both species. In 2009, three independent groups identified the essential function of the transcription factor (TF) Bcl6 for Tfh cell development and

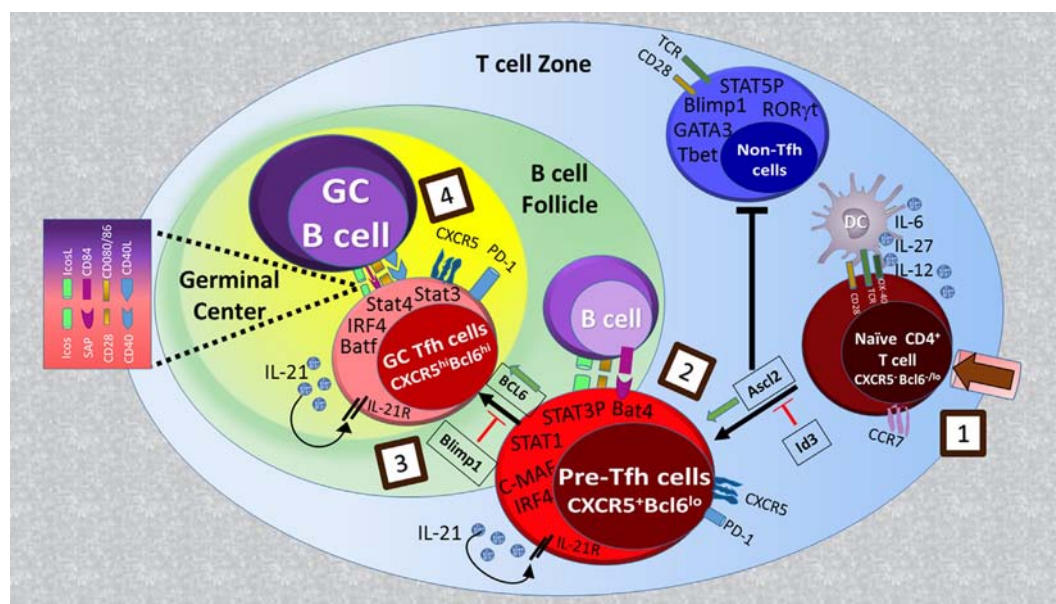


FIGURE 1 | Regulatory signaling for T follicular helper (Tfh) cell development: naïve CD4⁺ T cell priming by MHC/antigen interaction on DCs (step 1) leads to the generation of CXCR5⁺Bcl6^{lo} pre-Tfh cells with increased activity of transcriptional factors such as Achaete-scute homolog 2 (Ascl2), signal transducers and activators of transcription (STAT)1, STAT3, IFN-regulatory factor 4 (IRF4), and Batf (step 2). Ascl2 expression in activated T cells orchestrates them to migrate toward B cell follicles by upregulation of CXC chemokine receptor 5 (CXCR5) expression and repressing IL-2R-Blimp1 pathway as well as Th1, Th2, and Th17 cell differentiation. Upon interaction with cognate B cells at T-B border (step 3), CXCR5⁺ T cells begin to upregulate B-cell lymphoma 6 protein (Bcl6) expression, which in cooperation with other transcription factors determines the final differentiation state of Tfh cells within germinal centers (step 4).

function (7, 26, 27). Since then Tfh cells have been recognized as a distinct lineage of T helper cells.

Studies in mice and humans show that Tfh cells are localized in lymphoid organs and are composed of subsets that differ in their localization, phenotype, and function (28). In mice, after priming with DCs in the T zones of secondary lymphoid organs, a fraction of naive CD4⁺ T cells acquire CXCR5, PD-1, and Bcl6 expression, downregulate CCR7, and migrate toward B cell follicles (**Figure 1**) (21, 29–32). These CXCR5⁺Bcl6⁺CD4⁺ T cells, called Tfh precursors (pre-Tfh), interact with antigen-presenting B cells and further differentiate to fully programmed GC Tfh cells, which provide help to B cells within GCs. GC Tfh cells can be distinguished from Tfh precursors by high expression levels of CXCR5 and PD-1 (28). Sustained expression of Bcl-6 in GC Tfh cells is essential for GC formation (7, 26, 27). The origin of Tfh cells is not restricted to naive cells, and there is some evidence suggesting that other Th subsets including Th1, Th2, Th17, and regulatory T cells (Tregs) may become Tfh cells in GCs (3). This is consistent with the heterogeneity in cytokine expression patterns among GC Tfh cells developed under different immunization protocols and by different types of infectious agents (33). In human tonsils, CXCR5^{lo}ICOS^{lo} pre-Tfh cells (or extrafollicular helper T cells) express multiple Tfh molecules including CD40L, IL-21, and CXCL13 but not Bcl6 and are localized outside of GCs where they help naïve B cells to become immunoglobulin-producing cells (34). By contrast, CXCR5^{hi}ICOS^{hi}PD-1^{hi} GC Tfh cells provide help to GC B cells and promote their survival, proliferation, and differentiation into immunoglobulin-producing cells (28). In human tonsils, GC Tfh cells contain subsets coexpressing Bcl-6 and ROR γ t, and Bcl-6 and T-bet (8). This suggests that other Th subsets may be able to differentiate into Tfh cells or that Tfh cells share the same developmental path with Th1 and Th17 cells as observed in mice (35, 36).

A CXCR5⁺ subset of CD4⁺ T cells can also be identified in the peripheral blood of mice and humans, subsequently referred to as circulating Tfh (cTfh) cells. Although these CCR7^{lo}PD-1⁺ cells express lower levels of ICOS and PD-1 and seldom express Bcl6 they are closely related to Tfh cells (37). Their differentiation depends on ICOS and Bcl6 but not SLAM-associated protein (SAP), suggesting that cTfh cells are primary Tfh precursors or early memory Tfh cells. The cTfh cell subset undergoes active Tfh differentiation into mature Tfh cells in secondary lymphoid organs upon antigen reencounter and their presence correlates with autoimmune diseases such as lupus and rheumatoid arthritis (RA) (37). In addition, based on the expression of CXCR3 and CCR6, human cTfh cells can also be subdivided into three main subsets, namely, CXCR3⁺CCR6[−] (Tfh1), CXCR3[−]CCR6[−] (Tfh2), and CXCR3[−]CCR6⁺ (Tfh17) cells (38, 39). Tfh2 and Tfh17 cells, but not Tfh1 cells, represent efficient B cell helper cells to regulate immunoglobulin isotype switching (38). A recent data showed that in addition to cTfh memory, Tfh cells could be local in the draining lymphoid organs and sustain B cell responses after reactivation (40). In contrast to cTfh cells, local memory Tfh cells promote plasma cell differentiation and could be released to the circulating memory compartment over time.

Tfh CELL DIFFERENTIATION

T follicular helper cell differentiation is a complex process, which is tightly regulated (41) (**Figure 1**). It begins during naïve CD4⁺ T cell priming by DCs in the T cell zone of secondary lymphoid tissues and continues through the first cognate T cell:B cell interaction at the T–B junction until Tfh cells differentiate into mature GC Tfh cells when they enter follicles (2, 3, 42, 43). At each of these stages, Tfh cell development is influenced by signaling pathways downstream of cell surface molecules including the T cell receptor (TCR), costimulatory molecules, and cytokine receptors leading to the activation of specific transcriptional machinery (2, 3, 42, 43).

Costimulatory Molecules

T follicular helper cell differentiation from naïve CD4⁺ T cell precursors is a multistep process which requires costimulatory signals. Positive [CD28, ICOS, SAP, glucocorticoid-induced tumor necrosis factor receptor-related protein (GITR), etc.] and negative [CTLA4, PD1, and B and T lymphocyte attenuator (BTLA)] costimulation works together with TCR signaling during Tfh cell development to guide activation, proliferation, differentiation, migration, survival, and effector functions (1–3, 33, 44). Imbalance between positive and negative costimulation signals leads to increased Tfh cell number and consequently to Tfh-driven autoimmunity (45, 46).

Positive Costimulation

CD28 and ICOS

CD28 and ICOS are two structurally and functionally related costimulatory molecules that are critical for Tfh cell differentiation (2, 47, 48). CD28, which is constitutively expressed on both naïve and resting T cells, specifically binds to its ligands CD80 or CD86 and regulates T-dependent B cell responses (49, 50). While previous studies suggest the overlapping function of CD80 and CD86 in antibody responses, during virus infection CD86 expression on B cells but not CD80 is critical for Tfh cell generation and function (51). CD28 signaling regulates (i) early key events of Tfh differentiation, especially the expression of PD-1, ICOS, OX-40, Bcl6, and CXCR5 (47); (ii) the late stage of Tfh differentiation as B7 ligand blockade during ongoing infection impairs Tfh cell response (52, 53); and (iii) Tfh cell survival (53).

ICOS, another CD28 family member, is highly expressed on Tfh cells and is critical for Tfh cell generation and GC formation (2, 48). Unlike CD28, ICOS does not regulate any of the early differentiation steps of naïve T cells into Tfh cells, particularly Bcl6 expression (47); however, ICOS is important to maintain the phenotype of already differentiated Tfh cells (47). Mice in which the ICOS–ICOSL interaction is disrupted as well as ICOS-deficient patients have fewer Tfh cells and smaller GCs (6, 47). ICOS exerts its costimulatory function through phosphoinositide 3-kinase (PI3K) signaling which results in the activation of Akt (47). Akt phosphorylates the transcriptional factor FOXO1 which thereby stays in the cytoplasm and becomes functionally inactive (47). This is critical because FOXO1 suppresses Tfh cell differentiation through the negative regulation of Bcl6 and positive regulation of Klf2 expression (15). Klf2 plays a negative role

in Tfh differentiation by binding to promoter regions of CXCR5, CCR7, CD62L, PSGL-1, and S1pr1, which leads to the suppression of CXCR5, relocation of Tfh cells from B cell follicles back to T cell zone and their phenotype reversion to non-Tfh cells (44). Besides the ICOS-Klf2 axis, ICOS promotes interaction of the p85a regulatory subunit of PI3K with osteopontin (OPN-i), followed by translocation of OPN-I into the nucleus, its interaction with Bcl6 and protection of Bcl6 from proteasome degradation; thus, sustain responses by Tfh cells (54).

SLAM-Associated Protein

SLAM-associated protein is an intracellular adaptor protein essential for the function of SLAM family receptors (SFRs) to regulate immune responses (55, 56). SFRs consist of nine members, four of which are highly expressed on Tfh and B cells: CD150/SLAM, CD229/Ly9/SLAMF3, CD84/SLAMF5, and NTB-A/Ly108/SLAMF6 (57–59). High intrinsic SAP expression in both human and mouse GC Tfh cells, but not in B cells, is important for the development of long-term humoral immunity: particularly formation of GCs, long-lived plasma cells, and memory B cells (57, 60, 61). Bcl6 is required for SAP expression in GC Tfh cells (62). SAP through SFRs regulates T:B cell adhesion, cytokine production, and TCR signaling strength (63). Patients with X-linked lymphoproliferative disease (XLP) caused by a SAP gene mutation as well as mice lacking SAP expression, display defects in GC Tfh cell generation and GC reactions (3, 55). While SAP-deficient CD4⁺ T cells express normal levels of CXCR5, ICOS, and Bcl6, they have an impaired ability to stably interact with cognate B cells and sustain GC reactions (58, 61, 64, 65). Thus, SAP is not required for early Tfh cell differentiation (Bcl6^{hi}CXCR5⁺), but is indispensable for sustained T:B cell interactions and the full polarization to GC Tfh cells (Bcl6^{hi}PD1^{hi}) mainly by the following mechanisms: (1) SAP binds phosphotyrosines of the SLAM immunotyrosine switch motifs (ITSM) and mediates positive signaling by recruiting Src family kinase Fyn and PKC θ (66); (2) SAP competes with the tyrosine phosphatase SHP-1 for Ly108 ITSM binding, signaling through which contributes to reductions of T:B contacts and to an impairment in GC Tfh cell generation and GC responses (58). In addition to regulating T:B adhesion, positive signaling through SAP and PKC θ regulates cytokine secretion by GC Tfh cells, particularly IL-4 (60, 67). Moreover, Sap/Ly108 engagement sustains the TCR signaling that is required for establishing the Tfh cell:B cell synapse and thus allowing Tfh cells to provide help to B cells (68).

Glucocorticoid-Induced Tumor Necrosis Factor Receptor-Related Protein

Glucocorticoid-induced tumor necrosis factor receptor-related protein (TNFRSF18, and CD357), a member of the tumor necrosis factor receptor superfamily, is expressed at high levels in Tregs and activated T cells (69). Activation of GITR by its natural ligand GITRL enhances proliferation and effector T cell responses and inhibits Treg mediated suppression (70, 71). GITR is highly expressed on Tfh cells compared with non-Tfh cells in the spleens of collagen-induced arthritis (CIA) mice (72). GITR signaling promotes Tfh cell expansion and survival. Administration of

GITR-Fc protein greatly reduces CIA severity by suppressing Tfh development and thereby humoral immune responses. In addition, in the chronic lymphocytic choriomeningitis virus (LCMV) infection model, a Tfh cell intrinsic role for GITR in sustaining Tfh cell responses and LCMV-specific antibody production has been identified (73). Thus, GITR signaling is considered as a positive regulator of Tfh generation (44).

Negative Costimulation

Programmed Cell Death-1

Programmed cell death-1 (PDCD1 and CD279) is a member of the CD28 superfamily which is transiently expressed by activated conventional T cells and delivers an inhibitory signal by engaging its ligands PD-L1/CD274/B7-H1 and PD-L2/CD237/B7-DC that are expressed on activated B cells, DCs, and macrophages (74). PD1 shows high and sustained expression on exhausted T cells, Tregs, Tfh, and T follicular regulatory (Tfr) cells (75–78). In addition to conventional T cells, PD-1 is expressed by B cells, natural killer cells, and myeloid cells as well (79, 80). Early studies utilizing complete knockouts of PD1, PD-L1, or PD-L2, or their respective blocking antibodies tried to address the role of the PD-1 pathway in controlling humoral immunity. Interestingly, some studies have shown attenuated humoral immune responses upon PD-1 signaling blockade, whereas others have found enhanced responses (74, 81–85). Later work using cell-type specific deletion of PD1 and its ligand helped to delineate the function of PD1 signaling in GC responses and gain insight into the individual role of PD1 in Tfr and Tfh cells and PD1 ligands in DCs and B cells in the regulation of humoral immunity (77). The PD-1–PD-L1 pathway plays an immunoregulatory role in limiting the differentiation and suppressive function of Tfr cells (77). PD-1 expression can also modulate Tfh differentiation and function. Tfh cells from aged mice express higher levels of PD-1 compared with Tfh cells in young mice; PD-1 blockade in aged Tfh cells restores Tfh cell function, suggesting a cell-intrinsic role of PD1 in Tfh cells (12, 86). Interestingly, PD-L1 expression on DCs, but not B cells, inhibits Tfh and Tfr cell differentiation (87).

Cytotoxic T Lymphocyte Antigen 4

Cytotoxic T lymphocyte antigen 4 is a key checkpoint in immune tolerance (88–92). While CTLA4 and CD28 share the same ligands CD80 and CD86, CTLA4 interacts with them with higher affinity and avidity compared with CD28 (48). The central tenet of CTLA4 function is to regulate CD28 signaling, since fatal multiorgan inflammation as well as increased antibody levels in CTLA4 KO mice are prevented by the blockade of CD80 and CD86 (93–95). CTLA4 is constitutively expressed in Tregs and upregulated after activation in conventional T cells and plays a key role in mediating Treg function and in controlling conventional T cells (48). In fact, a Treg-specific deletion of CTLA4 recapitulates the phenotype of germline CTLA4 KO mice including increase in antibody production, indicating the role of CTLA4 in Tregs to control B cell responses (96). Recently, multiple groups assessed the role of CTLA4 in B cell responses and identified the function of CTLA4 on multiple T cell subsets including Tfh, Tfr,

and Tregs in regulating humoral immune responses (13, 97). While it was suggested that CTLA4-dependent suppression is the primary mechanism used by Treg and Tfr cells to control Tfh cell development and humoral immunity *via* CTLA4-dependent downregulation of CD80 and CD86 on B cells, Foxp1-dependent CTLA4 expression on non-Treg CD4⁺ cells has cell-intrinsic and negative regulatory functions in Tfh cell differentiation, maintenance, and function (13). CTLA4 controls Tfh cell differentiation by regulating the degree of CD28 engagement (52).

B and T Lymphocyte Attenuator

B and T lymphocyte attenuator (CD272) is an inhibitory receptor expressed on T and B cells that binds TNFR family member herpesvirus entry mediator and attenuates T and B cell activation and effector functions (98–100). Mice lacking BTLA exhibit increased antigen-specific IgG responses and with age gradually develop autoimmune hepatitis-like disease and autoantibody production to nuclear antigens (101), suggesting that BTLA negatively regulates humoral immune responses. BTLA is highly expressed in CXCR5⁺ Tfh cells compared with conventional CXCR5⁺ CD4⁺ T cells. While Tfh cell development is not affected in BTLA-deficient mice, BTLA expression in Tfh cells but not in B cells is critical to control GC B cell development and antigen-specific IgG2a and IgG2b production (102). Moreover, BTLA controls Tfh-mediated B cell responses by suppressing IL-21 production (102).

Cytokines

Along with antigen and costimulation signaling, specific cytokine-dependent cues play a central role in governing naive CD4⁺ T cell differentiation into specific effector T helper cell subsets. For example, IL-12 and IFN γ promote Th1 differentiation, whereas IL-4 drives Th2 differentiation (42). In addition, IL6 and IL-21 in combination with TGF β induce Th17 differentiation (42). There are multiple cytokines that exercise either positive or negative roles at different stages of Tfh development (1, 2). However, cytokine-dependent Tfh cell formation varies between mice and humans (1, 42). Particularly, while TGF β signaling opposes Tfh development in mice, it is required for human Tfh cell development (42).

Cytokines That Support Tfh Cell Formation in Mice and Humans

IL-6, IL-21, and IL-27

IL-6, IL-21, and IL-27 have all been implicated in Tfh cell development, although with differing roles (1, 2, 6, 7, 103, 104). IL-6 is mainly derived from activated B cells, DCs, and follicular DCs and is required in the initial stage of Tfh cell formation by inducing Bcl6 and IL-21 expression (5, 103, 105, 106). Mice deficient in IL-6 or IL-6R show reduced or delayed Tfh cell formation due to impaired signaling through STAT3 and STAT1 (5, 107). In addition, at the late stage of chronic viral infection, IL-6 derived from activated follicular DCs is crucial for maintenance of Tfh cell by upregulation of Bcl6 and viral control (3). Similar to mice, in humans, IL-6 derived from circulating plasmablasts is also a potent inducer of Tfh differentiation (108). IL-21 is primarily

produced by select CD4⁺ T cells including Tfh, Th17 cells, and natural killer T (NKT) cells and plays a more prominent role in sustaining Tfh cell identity and function (6, 7, 18, 36, 109). IL-21- and IL-21R-deficient mice display reduced numbers of Tfh cells after antigen immunization suggesting an autocrine role for IL-21 in the maintenance and augmentation of Tfh cell programming (6, 110). However, in mice deficient either in IL-6 or IL-21 signaling, Tfh cell development is only partially compromised, indicating that these cytokines may play redundant roles in Tfh cell development (5, 103). In fact, loss of both cytokines significantly diminished Tfh cell numbers compared with an IL-6 or IL-21 deficiency alone (5, 103). However, an IL-6/IL-21 deficiency does not cause the complete absence of Tfh cells, suggesting an existence of IL-6 and IL-21-independent mechanisms for Tfh cell generation. In fact, it has been reported that the cytokine IL-27 contributes to Tfh cell maintenance by promoting IL-21 expression (104). Mice deficient in IL-27 signaling show reduced IL-21 expression, Tfh cell number, and GC activity (104). Similar to mice, DC-derived IL-27 is critical for the induction of Tfh cell polarization, IL-21 secretion by Tfh cells, and Tfh-dependent production of IgG by B cells (111). In addition to IL-21 induction, it has been suggested that IL-27 may play an important role in Tfh cell development by antagonizing IL-2 signaling, which negatively regulates Tfh cell development (10, 112).

TGF- β , IL-12, IL-23, and Activin A

Recent data suggest that different groups of cytokines support Tfh cell formation in humans, with prominent roles for TGF- β , IL-12, and IL-23. While IL-12 and IL-23 are capable of inducing IL-21 expression in naive CD4⁺ T cells from human tonsils and peripheral blood, only IL-12 could augment expression of CXCR5, ICOS, CD40L, and Bcl6, thus IL-12 is likely to act at an early stage of human Tfh cell development. B cells co-cultured with IL-12-primed CD4⁺ T cells produce antibodies which in part is dependent on the expression of CD40L (113). In support of the importance of IL-12 and IL-23 for Tfh development, individuals with mutations disrupting the function of IL-12R β 1 (receptor for IL-12 and IL-23) have fewer circulating and GC Tfh cells and memory B cells. In mice, acting through STAT4, IL-12 induced a transitional stage in Tfh–Th1 cells, which express IL-21 and Bcl6. However, the IL-12–STAT4 pathway also promotes Tbet expression, which ultimately represses Bcl6 expression and Tfh cell programming. Since IL-12 is also linked to the generation of human Th1 cells, additional factors may also contribute to human Tfh cell generation. Surprisingly, TGF- β acts as an important cofactor for the early differentiation of human Tfh cells, but not in mice (7, 8, 114). TGF- β synergizes with IL-12 and IL-23, activating STAT4 and STAT3, and promoting Tfh cell development through induction of Tfh key molecules including CXCR5, ICOS, IL-21, Bcl6, Batf, c-Maf, and the downregulation of Blimp-1 expression (8). Recently, Activin A was identified as a novel inducer of Tfh cell programming in human and non-human primate cells, but not in mice by upregulating CXCR5, CXCL13, and PD-1 expression and repressing CCR7 and Blimp-1 expression (9). Activin A, in combination with IL-12, promotes the generation of Tfh like cells that have high expression levels

of CXCR5, Bcl6, PD-1, LAMF1, CXCL13, IL-21, LIF, LTA, and TNF (9). Activin-A-induced Tfh programming is dependent on signaling *via* SMAD2 and SMAD3 (9).

Cytokines Which Inhibit Tfh Cell Formation

IL-2, IL-10, and IL-7

The IL-2/STAT5 axis is shown to be inhibitory for Tfh cell formation. In an *in vivo* mouse model of influenza infection, it was shown that exogenous administration of IL-2 suppresses Tfh cell differentiation, GC formation, and neutralizes antibody production (10). T cell-intrinsic expansion of Tfh cells is mediated by loss of IL-2R α (115, 116). Mechanistically, activation of STAT5 by IL-2 enhances Blimp-1 expression and prevents binding of STAT3 to the Bcl6 locus (10). In addition, IL-2 inhibits the expression of IL-6 receptor α -chain (IL-6R α) and to a lesser extent, gp130 and thus negatively regulates Tfh differentiation (117). Moreover, a recent study depicts that IL-2 drives cells toward Th1 rather than Tfh differentiation through the activation of Akt and mTORC1 kinase (118). IL-7 negatively regulates Tfh differentiation by activating STAT5 and thereby repressing Bcl6 and CXCR5 (11). An additional study showed that IL-10, which has traditionally been viewed as a costimulator of antibody production, could also inhibit antibody responses indirectly by suppressing a subset of Tfh cells that produce IL-17 and IL-21 (119).

Transcriptional Regulation of Tfh Cells

The differentiation of naïve CD4⁺ T cells into Tfh cells is regulated by coordinated interplay between cell-extrinsic factors and cell-intrinsic transcriptional networks. Since the discovery of Bcl6 as a key factor for Tfh differentiation, several other transcriptional factors that either support [IFN-regulatory factor 4 (IRF4), c-Maf, Batf, STATs, Ascl2, TCF1, LEF1, etc.] or oppose [FOXO1, FOXP1, BLIMP-1, STAT5, KLF2, and peroxisome proliferator-activated receptor gamma (PPAR γ)] Tfh cell development, migration, and function have been defined (1–3). A dynamic balance between these multiple transcriptional factors is the main determinant of normal Tfh development and immunity, since dysfunction of negative transcriptional machinery triggers autoimmunity (120, 121).

TFs Positively Influencing Tfh Cell Differentiation

B-Cell Lymphoma 6 Protein

The Bcl6 has been recognized as a key transcriptional factor for Tfh cell development and for efficient GC responses (7, 26, 27). Expression of Bcl6 is driven by CD28 and IL-6/IL-21-STAT1/STAT3 signaling (47). Bcl6 overexpression leads to the upregulation of PD-1, CXCR5, CXCR4, and SAP which are essential for Tfh cell function in T and B cell interactions (106, 115). As a transcriptional repressor, Bcl6 functions to suppress genes that controls Tfh cell development by the following mechanisms: (i) Blimp-1 expression; (ii) genes encoding proteins that controls migration including EBI2, CCR7, CCR6, S1PR1, and PSGL1; (iii) Klf2 expression; (iv) genes that support Th1 (IFNGR1, T-bet, and STAT4), Th2 (Gata3), and Th17 (IL-17 and ROR γ t) cell development (7, 26, 27, 122–126).

c-Maf

c-Maf is a bZIP transcriptional factor that plays an important role in the regulation of cytokine production and Th2, Th17, and Tfh cell differentiation (87). Both Th17 and Tfh cells have higher expression of c-Maf, and loss of c-Maf in T cells results in a defect in IL-21 production and fewer Th17 and Tfh cells (36). Recent data further indicate an important and non-redundant role for c-Maf in the initiation of Tfh cell development and T-cell-mediated humoral responses. Loss of c-Maf expression in T cells leads to the decreased expression of key Tfh molecules, such as BCL6, CXCR5, and PD1 (127).

Batf

Batf is basic leucine zipper protein in the AP-1 family which was originally implicated in Th17 differentiation through direct regulation of transcription of ROR γ , IL-21, and IL-22 (128). While Batf expression is moderately increased in Tfh cells compared with other T helper subsets, Batf is an essential component for Tfh development through combined regulation of Bcl6 and c-Maf (129, 130). Batf-deficient mice fail to generate Tfh cells, and Bcl6 and c-Maf overexpression in Batf-deficient T cells improves Tfh cell development but not to the level of Batf reconstitution, suggesting that additional targets are required for complete Tfh cell induction (130). In addition, the Batf/IRF4 complex in cooperation with STAT3 and STAT6 is required for IL-4 expression in Tfh cells (120).

IFN-Regulatory Factor 4

Together with the well-known function of IRF4 in Th2, Th17, Th9, and Treg differentiation as well in plasma cell maturation (131–139), IRF4 also plays a critical role in Tfh cell development and GC formation (140). Mechanistically, IRF4 may cooperate with other transcriptional factors that determine T cell fate decision: (i) IRF4 interacts with Jun and Batf to form an IRF4–Jun–Batf complex that binds to AP-1–IRF4 composite elements (141); (ii) binds with STATs (142), and Bcl6 (143). Recently, it has been acknowledged that TCR signaling strength controls IRF-4 concentration and consequently cell fate choice between Bcl6-expressing Tfh and Blimp-1 expressing Teff cells (144). Increased TCR signaling leads to elevated IRF4 levels that function to coordinate Teff cell fate choice at the expense of Tfh cell fate (144).

Signal Transducers and Activators of Transcriptions

There are several members of the STAT family including STAT1, STAT3, and STAT4 that contribute to Tfh cell development (1, 6, 14, 106). It has been reported that STAT1 is required for early Tfh differentiation (107). Besides, IL-6 signaling during Tfh differentiation is mediated by both STAT1 and STAT3 TFs (107). It has also been noted that STAT1 directly regulates expression of key Tfh genes including Bcl6, CXCR5, and PD-1 by binding to their promoter loci (145). IL-6-STAT3 and IL-21-STAT3 signaling can promote Tfh cell differentiation by inducing Bcl6 expression (6, 7, 146, 147). STAT3 cooperates with the Ikaros zinc finger TFs, Aiolos, and Ikaros, to regulate Bcl6 expression (148). IL-6-mediated STAT3 activation restricts IL-2R α expression to

limit Th1 cell differentiation (107). In humans, functional STAT3 deficiency compromises Tfh cell generation (149). STAT4 can promote the expression of Bcl6 and the classical Tfh cell cytokine IL-21 in both mouse and human Tfh cells *in vitro* (35, 150). However, continued IL-12-driven Stat4 signaling can decrease the expression of Bcl6 and IL-21 and strengthen T-bet-derived Th1 differentiation at the expense of Tfh cells (35). Interestingly after acute viral infection, T-bet is co-expressed with Bcl6 in Tfh cells and is required alongside STAT4 to coordinate IL-21 and IFN- γ production in Tfh cells and for promotion of the GC response (151).

Notch1 and Notch2

Notch proteins belong to the family of evolutionary conserved transmembrane-bound receptors and play an important role in CD4⁺ T helper cell differentiation and/or function including Tfh cells (152). Mice with T-cell-specific deletions of Notch1 and Notch2 display impaired Tfh cell differentiation, IL-4 secretion by Tfh cells and GC reactions after immunization with T-dependent antigens or infection with parasites (153). Notch1- and Notch2-deficient Tfh cells express reduced levels of Tfh-associated molecules (CXCR5, PD-1, BTLA, and Bcl6), but normal levels of ICOS and increased Blimp-1 expression (153). Notch receptors 1 and 2 are required for Tfh cell generation and IL-4 expression by Tfh cells, but are dispensable for Th2 cell differentiation in response to parasitic helminth infection. Thus, Notch signaling is an important checkpoint in the bifurcation between Tfh and Th2 cell-driven hallmarks of type-2 immunity (154).

NFAT

Nuclear factor of activated T cells 2 (NFAT2) is highly expressed in Tfh cells, NFAT2 deficiency in T cells leads to enhanced GC reactions due to the impairment of Tfr cells to upregulate CXCR5 but not Tfh cells (155). A loss of both NFAT1 and NFAT2 in CD4⁺ T cells leads to impaired GC reactions due to reduced Tfh cell differentiation and decreased expression of proteins such as ICOS, PD-1, and SFRs which are important players in T/B interactions and B cell help (156).

Achaete-Scute Homolog 2

Achaete-scute homolog 2, a bHLH-domain-containing TF, is selectively upregulated in Tfh cells and initiates Tfh cell development (157). Overexpression of Ascl2 can lead to a substantial induction of CXCR5 expression, but not Bcl6 and downregulation CCR7 expression *in vitro*, as well as accelerated T cell migration to the follicles and Tfh cell development *in vivo* in mice (157). Ascl2 inhibits expression of Th1 and Th17 signature genes (157). Ascl2 deletion as well as inhibition of its function with E-protein inhibitor Id3 leads to a total impairment of Tfh cell development and GC response (157).

T Cell Factor 1 (TCF-1) and LEF-1

T cell factor 1 is highly expressed in Tfh cells (158–160). TCF-1 plays an important role in the initiation of Tfh cell differentiation and the effector function of differentiated Tfh cells, as TCF-1 deficiency results in reduced generation of Tfh cells and impairs

their function to provide B cell help (159, 160). Similar to TCF-1, LEF-1 is also known for its essential role in early Tfh cell development (161, 162). LEF-1 and TCF-1 are upstream of Bcl6 induction and directly target Tfh signaling molecules (Bcl6, IL-6R, gp130, and ICOS) to promote Tfh cell differentiation (158). TCF-1 also suppresses Blimp-1 and IL-2Ra expression (159).

Early Growth Response Gene 2 (EGR2) and EGR3

Early growth response gene 2 and EGR3 can directly regulate the expression of Bcl6 and differentiation of Tfh cells (163).

Bob1

It has been reported that B-cell-specific octamer-binding protein 1, Bob1 in cooperation with TFs Oct1/Oct2 can directly bind to Bcl6 and BTLA promoters and promote their expression and Tfh cell development (164). However, at the same time, other groups have reported that the function of Bob1 is to mainly restrict the cellular frequency of Tfh cells (165, 166).

NF- κ B1

It has been reported that NF- κ B1 promotes Tfh cell responses by facilitating CXCR5 expression but no other Tfh-related molecules (Bcl6, IL-21, and PD-1), and the NF- κ B1-deficient T cells partially lose their ability to provide help to B cells *in vivo* (167). In addition, the non-canonical NF- κ B pathway may also play an essential role in Tfh development through regulation of ICOSL expression in B cells (168).

TFs Negatively Influencing Tfh Cell Differentiation

FOXO1 and FOXO1

Early studies suggest that a decreased expression of FOXO1 either because of increased expression of ICOS (15) or because of ITCH-mediated degradation (169) may increase Tfh cell differentiation. Analysis of mice with a specific deletion of Foxo1 in T cells revealed the requirement for Foxo1 in the suppression of Bcl6 expression and Tfh cell differentiation (15). In addition, enforced nuclear localization of Foxo1 prevents Tfh cell differentiation (15). However, Foxo1 is required during final differentiation to GC Tfh cells as Foxo1 deficient GC Tfh cells are substantially reduced (15). Foxp1 is an additional negative regulator of Tfh cell development. It mainly plays a role in dampening ICOS and IL-21 expression by regulating CCR7 and CTLA4 expression (170).

Krüppel-Like Factor 2

The TF, Klf2, regulates naïve T cell trafficking to secondary lymphoid tissues by promoting the expression of CD62L and S1PR1 (16). KLF2 expression impairs Tfh cell differentiation, whereas ablation of KLF2 expression enhances Tfh cell differentiation (171). This effect is related to the capacity of KLF2 to promote the expression of genes that oppose Tfh cell differentiation (Blimp-1, Gata3, and T-bet) and to repress the transcription of CXCR5 (171).

Blimp1 and STAT5

Blimp1, which is encoded by *Prdm1*, is a transcriptional repressor that has the ability to inhibit Bcl6 expression in B and T cells (122).

Blimp-1, induced by IL-2/IL-7 and STAT5 signaling, suppresses expression of Bcl6 and other Tfh-associated genes including CXCR5, c-Maf, Bcl6, Batf, and IL-21, thus preventing Tfh cell differentiation (14, 172).

Peroxisome Proliferator-Activated Receptor Gamma

Peroxisome proliferator-activated receptor gamma is a TF that regulates lipid and glucose metabolism (173). One-year-old T-cell-specific PPAR γ -deficient mice exhibited a moderate autoimmune phenotype with increased Tfh cells, GC B cells, glomerular inflammation, and enhanced autoantibody production. Mechanistically, PPAR γ by stabilizing the activity of Ikb α , Foxo1, and Sirt1 negatively regulates Bcl6 and IL-21 to inhibit Tfh cell differentiation and GC formation (173).

OTHER FOLLICULAR T CELLS

Several types of follicular T cells that have been found in GCs and characterized such as Tfr cells, follicular regulatory CD8⁺ T cells (CD8 Tfr), natural killer T follicular helper (NKTfh) cells, and follicular CD8⁺ T cells (fCD8).

Tfr Cells

T follicular regulatory cells, a newly identified subset of Tregs, have been found in GCs where they control GC responses (75–77, 86, 174–176). Tfr cells originate from thymic-derived Foxp3⁺ T cells as well as from Foxp3[−] precursors rather than from Tfh cells (78, 177). They are different from Tfh cells and Tregs: on the one hand, Tfr cells express large amounts of Tfh-related factors including CXCR5, PD-1, Bcl6, CXCL13, and ICOS; on the other hand, they share numerous molecules that are expressed by Tregs, such as GITR, CTLA4, IL10, CD25, and Foxp3 (75, 76, 178).

T follicular regulatory cells similarly possess a multistage and multifactorial differentiation process (176) that requires CD28 and ICOS signaling (76, 77), Sap-dependent interaction with B cells (76), and expression of Bcl6 (178). TF NFAT2 contributes to the initial upregulation of CXCR5 in Tfr cells (155). In addition, recently it has been noted that the mTORC1–STAT3–TCF-1–Bcl6 axis and TRAF3 are essential for Tfr differentiation (179, 180). However, there are also some signals which inhibit Tfr-cell differentiation and function: PD-1, CTLA4, Blimp1, and helix-loop-helix proteins ID2 and ID3 (77, 78). In addition, the cytokine IL-21 can suppress Tfr cells through the upregulation of Bcl6 expression and down-regulation of CD25 (103, 181, 182).

T follicular regulatory cells act to limit excessive GC responses by acting on both Tfh and GC B cells (13, 77, 86, 183). Moreover, Treg suppression is not limited to GC B cells and occurs at various steps during B cell differentiation, from B cell activation to class-switched B cells and plasma cells (78). The effect of Tfr cells to modulate GC reaction could be through several potential mechanisms. Tfr cells could suppress Tfh cells through CTLA-4, by dampening expression of CD28 ligands on GC B cells (13). Two immunosuppressive cytokines, IL-10 and TGF- β , may mediate the immune suppressive functions of Tfr within GCs (114, 119, 176, 184); however, a recent research showed that IL-10

is important for B cell survival and proliferation; thus, a detailed mechanism remains to be investigated (185). Interestingly, Tfr cells potentially have effects on antibody affinity, and their suppressive function could result in the selection of the highest affinity antigen-specific antibody and in the selection of higher affinity memory B cells (78).

Follicular Regulatory CD8 T Cells (CD8 Tfr)

In contrast to Foxp3⁺CD4⁺ Tregs, mouse CD8 Tregs do not constitutively express Foxp3 in the thymus and periphery (186), and the CD8⁺Foxp3⁺ T cells do not comprise CD8 Treg population due to lack of suppressive activity (187). Similarly, most of the human CD8 Tregs also lack Foxp3 (188). In mice, a specific subset of Qa-1-restricted CD8 Tregs with high expression levels of CXCR5 (named as CD8 Tfr) were found to possess the ability to limit GC size and prevent autoimmune disease in mice (189). Tfh cells are one of main targets of CD8 Tfr cells (189). In autoimmune-prone mice, CD8 Tfr cells can suppress the expansion of Tfh cells as well as autoantibody production (190). Recent data indicate on the importance of the TF STAT4 for CD8⁺ Tfr development, maintenance, and function toward Tfh and plasma B cells (191). Moreover, CD8 Tfr cells expressing IL-2R β are also shown to inhibit CD8 T cell function in an IL-10-dependent manner (192). Recently, cells with a CD8 Tfr phenotype (CD3⁺CD8⁺CXCR5^{hi}CD44^{hi}) have been identified in humans (193). In chronic CIV infection, CD8 Tfr cells localized in the follicles exhibit enhanced Tim-3 and IL-10 expression, but express less perforin compared with CD8 T cells. CD8 Tfr cells modestly limit HIV replication in Tfh cells by impairing IL-21 production *via* Tim-3 and inhibit B cell function (194). In addition, it has been reported that the KIR⁺CD8⁺ cells (KIR, killer cell immunoglobulin-like receptor, functional homolog of murine Ly49) exert inhibitory activity on CD4⁺CXCR5⁺ Tfh target cells in humans (193).

NKTfh Cells

Recently, a subset of invariant NKT cells, recognized as follicular helper NKT cells (NKTfh cells), was discovered (195). NKTfh cells express CXCR5, PD-1, and Bcl-6 and support B cell responses (196). Similar to the development of conventional Tfh cells, the formation of NKTfh cells is dependent on CD28-mediated cognate interactions with B cells and Bcl6 expression (196). Studies utilizing CD4⁺ T cell-specific loss of Bcl6 determined that both Tfh and iNKTfh cells contribute to B cell help (197). However, unlike Tfh-derived B cell responses, those driven by NKTfh cells have no potential to generate long-lived plasma cells and memory B cells (196). NKTfh cells also possess the ability to boost memory B cell responses to T-dependent antigens but not T-independent lipid antigens (198). In addition, the NKTfh cells can induce limited GC B cell responses in the absence of CD4⁺ cell help (199). Further studies are still needed for a deeper understanding of the mechanisms of NKTfh cells in immunity and their roles in disease.

Follicular CD8⁺ T Cells (fCD8)

In contrast to the previous opinion that CD8⁺ T cells are restricted to extrafollicular areas (200), recent studies have showed that the

CD8⁺ T cells from human and non-human primates possess the ability to migrate to the lymphoid follicles and GCs to support B cells (201). CD8⁺ T cells localize in human tonsil follicles and possess follicular helper-like characteristics including high expression of Bcl6, CXCR5, ICOS, PD-1, CCR5, CD27, CD28, CD69, and CD95, but do not express CCR7, Blimp-1, Tim-3, and CD244 and are named as follicular CD8⁺ T cells (fCD8) (201–206). The current evidence suggests a crucial role of fCD8 in controlling intracellular pathogens and malignancies through the production of various cytokines (IFN- γ , TNF- α , and MIP1 β) in LCMV-infected mice, HIV-infected individuals, and cancer patients (203–205, 207–211). These cytokines activate APCs, promote polarization of naïve CD4⁺ T cells to Th1 cells, sustain activation of CD8⁺ T cells; thus, contributing in the control of viral infection and tumor growth. In addition, similar to Tfh cells, IL-21-producing fCD8 cells promote Ag-specific antibody responses by stimulating B cells, as well as generating and maintaining follicles and GCs (201).

THE ROLE OF Tfh CELLS IN DISEASES

The main function of Tfh cells is to control clonal selection of GC B cells and support B cell immunoglobulin synthesis, isotype switching, and somatic hypermutations. Pathological B cell activation and the production of autoantibodies is a hallmark of the defective immune response that accompanies autoimmunity. In the sections below, we discuss the role of Tfh cells in various disease settings (Figure 2).

Systemic Lupus Erythematosus (SLE)

Observations in animal models and in humans provide strong evidence that Tfh cells are important players in SLE pathology. Patients with SLE have an increased number of cTfh cells, which positively correlates with autoantibody titers (212). Furthermore, the proportion of peripheral blood T cells expressing ICOS is higher in patients with active SLE disease than in patients with inactive disease or in controls which probably leads to enhanced autoantibody production upon activation *via* ICOS–ICOSL interactions in these patients (45, 213). Production of high levels of IL-21 is a hallmark of Tfh cells and multiple studies have shown an increased frequency of CD4⁺IL-21⁺ T cells in SLE patients that was associated with disease severity (214). An increase in Tfh cells is also associated with a shift in the Th17/Treg populations with increases in Th17 cells and decreases in Tregs as well as an elevation of IgG⁺ class-switched memory B cells leading to a more inflammatory environment as observed in SLE (214–219). It is worthy to note that Tfh cells not only affect priming in secondary lymphoid tissues but can also impact local B cell activation and expansion. For example, within the tubulointerstitium of patients with lupus nephritis, Tfh-like cells are organized with B cells in structures resembling GCs (220, 221) suggesting that Tfh cells may also play a role in various complications that are associated with autoimmune pathologies.

Mice that have a single-amino-acid mutation in Roquin1 (a negative regulator of ICOS mRNA stability) demonstrate a spontaneous lupus-like phenotype that is accompanied by

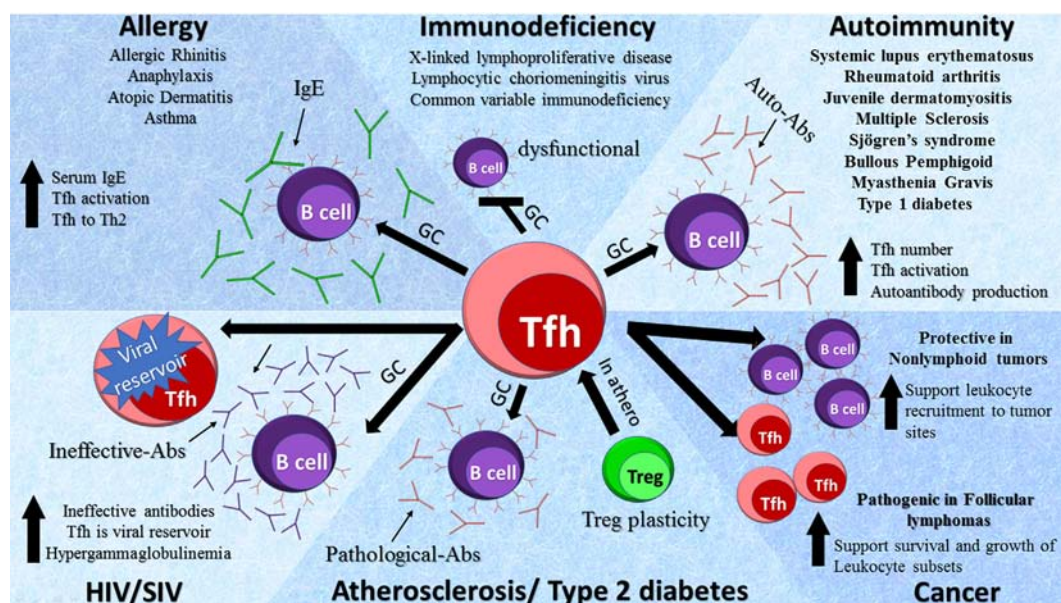


FIGURE 2 | Role of T follicular helper (Tfh) cells in diseases. Tfh cells are involved in a variety of pathologies by diverse mechanisms as depicted in the schematic diagram. Elevated Tfh activity leads to an increase in germinal center (GC) formation, activity, and subsequent production of auto-Abs in autoimmune diseases. In primary immunodeficiencies, a decrease in Tfh cells has been detected with dysfunctional GC formation, whereas in acquired immunodeficiencies such as HIV and SIV an increase in Tfh cells serves as viral reservoirs. In atherosclerosis regulatory T cells (Tregs) transition to Tfh cells *via* GCs leading to the production of pathological Abs. In allergy, there is an increase in Tfh cell activation and GC formation causing the production of IgE. The role of Tfh cells in cancers is complex and dependent on the types of tumors.

elevated numbers of Tfh cells expressing higher level of ICOS, IFN- γ OX40, and IL-21 and activated phenotypes of GCs (28, 222). Importantly, manipulation of Bcl6 expression and thus Tfh cell generation or adoptive transfer of lupus-associated Tfh cells from Roquin^{san/san} mice into healthy recipients induces the formation of GCs, highlighting a contributing role of Tfh cells in SLE (223). Another interesting model to study lupus-related pathophysiology is the BXSB mouse model, which displays lymphoid hyperplasia, monocytosis, immune complex-mediated glomerulonephritis, and an aberrant IL-21-dependent Tfh response (224). The MRL/lpr mouse model displays defective Fas signaling (225), which is characterized by high levels of autoantibodies and extrafollicular Tfh like cells that are dependent on ICOS, Bcl-6, and IL-21 signaling. The pathological functions of these T cells support extrafollicular B cell differentiation and plasmablast maturation (226). While increasing evidence suggests the importance of an extrafollicular Tfh-dependent response in the murine models of SLE (227–230), the importance of Tfh cells and extrafollicular sites in SLE patients is not well defined due to limitations in obtaining non-circulated follicular-resident Tfh cells.

There have been several relatively successful attempts to reduce the severity of SLE in humans *via* blockade of Tfh-cell differentiation and activity. Early results from SLE therapies targeting T cells showed that patients had reduced serum anti-dsDNA titers, but presented minimal reduction of protective antibodies, increased complement markers, and reduced nephritis scores (231). Studies using monoclonal antibodies against ICOS-L inhibited the development of Tfh and GC B cells resulting in decreases in anti-dsDNA Igs and improved kidney function. For years, the main therapy for SLE has been broad spectrum immunosuppressants; however, a growing body of work shows that Tfh cells may be a more precise and attractive target for treating SLE.

Sjögren's Syndrome

Increased numbers of human peripheral blood CXCR5⁺ICOS⁺, CXCR5⁺PD-1⁺ Tfh cells and enhanced GC formation positively correlate with autoantibodies titers and severity of the primary Sjögren's syndrome (pSS) (227–230). IL-6 levels are increased in the serum, tears, and salivary gland epithelial cells of patients with pSS. Interestingly, co-cultures of salivary gland epithelial cells with T cells induce Tfh cell differentiation (3, 232) suggesting a critical role for epithelial cell-derived IL-6 in Tfh cell differentiation in pSS. There are several treatment options for patients with pSS that target Tfh cell functions and therefore the humoral response. Abatacept is a fusion molecule combining CTLA-4 with IgG Fc that binds to CD80/86 and consequently impairs CD28-mediated T cell costimulation (233). It has been shown that abatacept inhibits T cell-dependent B cell activation either *via* DC-defective Th activation or *via* blockade of T/B cell interactions (234). Abatacept treatment in pSS patients reduces circulating numbers of ICOS⁺ cTfh cells resulting in an attenuated Tfh cell-dependent B cell hyperactivity (235) providing a promising therapy for pSS and some other autoimmune pathologies.

Juvenile Dermatomyositis (JDM) and Autoimmune Myasthenia Gravis (MG)

Serum autoantibodies can be found in up to 70% of patients with JDM. Furthermore, these patients display changes in cTfh cells subsets with a decrease in cTfh1 cells, and an increase in the cTfh2 and activated memory cTfh17 cell subpopulations; resulting in an overall increase in cTfh subsets with efficient helper functions. Significantly, these changes in the composition of cTfh cell subsets positively correlate with disease activity and the frequency of circulating plasmablasts (38).

Elevated levels of circulating CXCR5⁺CD57⁺, CXCR5⁺ICOS^{hi}, and CXCR5⁺PD1^{hi} CD4⁺ T cells have also been reported in patients with MG (236). Functional studies demonstrate that cTfh cells from MG patients support autoantibody production, and thus, contribute to the development of disease. Interestingly, Tfh1 and Tfh17, but not Tfh2 cells, were found to be the major secretors of IL-21 (237). Thus, alterations in the composition of peripheral blood memory Tfh1, Tfh17, and Tfh2 subsets seems to be one of the distinct features of several autoimmune diseases including SLE (238), Sjögren's syndrome, multiple sclerosis (239), and MG (237). While an increasing body of evidence reports a shift in the Tfh1/Tfh2/Tfh17 balance in the peripheral blood of patients with autoantibody-mediated pathologies, it is not clear whether the same shift in the Tfh subsets occurs within the inflamed tissues and GCs in the secondary lymphoid tissues. It would be important to identify molecular mechanisms and microenvironmental stimuli that direct a preferential repopulation of Tfh17 and Tfh2 cells in autoimmune settings.

Rheumatoid Arthritis

One of the other examples for the implication of Tfh cells in autoimmune disease setting is RA, which is characterized by high levels of autoantibodies and abnormal GC B cell responses, which contribute to inflammation in the joints. Tfh cells have been detected in the synovial tissue of patients with RA, and along with IL-21 are found in higher frequencies in the periphery (240–242). The increase in Tfh cells in RA patients is positively correlated with elevated serum level of anti-CCP antibodies and consequently with disease severity (240). In animal models, a T cell-specific CXCR5 deficiency results in a significant reduction in GC formation, decreased levels of collagen-specific IgG1 antibodies and a resistance to CIA induction (243). As Tfh cells play a significant role in the progression of RA, therapeutic targeting of Tfh cells could be a valuable option for treating patients with RA.

Type 1 Diabetes (T1D)

Type 1 diabetes is caused by the autoimmune destruction of insulin-producing β -cells in the pancreas. T1D patients have higher frequencies of circulating CD4⁺CXCR5⁺ICOS⁺Tfh cells and higher levels of IL-21 which positively correlates with an increase in plasmablasts, serum autoantibodies, and C-peptide levels (244–246). It is possible that Tfh cells may play a critical role in the initial stages of T1D with an increase of activated CXCR5⁺PD-1⁺ICOS⁺ cTfh cells being found in both children with newly diagnosed T1D and in children at late stages of preclinical

T1D, characterized by impaired glucose tolerance (247, 248). Data from animal models also show that transfer of Tfh cells from a diabetic to a control animal induces elevated blood glucose levels and increased T cell infiltration into islets (244). Furthermore, Roquin^{san/san} mice that have overreactive Tfh cells display dramatically accelerated T1D induction. Collectively, there is direct evidence for a pathogenic role for Tfh cells in progression to T1D. Importantly, the increase in activated cTfh cells is strongly associated with positivity for multiple autoantibodies and could be used as a biomarker for the identification of a subgroup of patients with an active setting of T1D.

Type 2 Diabetes (T2D)

Evidence indicates a key contribution of the immune response in the manifestation of chronic low grade inflammation under the conditions of adipose tissue inflammation, islet β -cell dysfunction, and T2D. While the role of macrophages, CD4⁺, CD8⁺ cells, and follicular B cells is firmly established, there is limited knowledge about the Tfh cell role in obesity and T2D. Recently, Zhou and colleagues found that non-obese T2D patients vs BMI-matched healthy subjects have higher IgG levels and Tfh cells that are highly enriched in IFN- γ , but not IL-4 and IL-17 (249). Interestingly, overweight T2D patients (BMI $\geq$ 24.0) had higher levels of cTfh and the balance of cTfh cell subsets was shifted toward the Th17 subtype (52). Importantly, patients with abdominal obesity had additional increases in cTfh compared with patients without abdominal obesity (52), suggesting that cTfh may play a critical role in the modulation of adipose tissue inflammation in obesity-induced T2D.

Atherosclerosis

Recent reports revealed a critical role for Tfh cells and consequently GC B cells in the development and progression of human and mouse atherosclerosis. Tfh cells support atherogenesis *via* the production of pathological Abs and the generation of highly active GCs. The loss of Tfh cells *via* a conditional knockout of *Bcl6* leads to a reduced atherosclerotic burden in atherosclerosis-prone mice (250, 251). Interestingly, a percentage of Tregs can switch the phenotype into pro-atherogenic Tfh cells but ApoAI can prevent Treg to Tfh cell conversion throughout atherosclerosis (250). It remains to be determined how atherosclerosis-prone conditions alter the memory pool of Tfh cells and how Tfh cells impact the processes of selection of high-affinity B cells and B cell memory development in atherosclerosis.

Allergy

Patients with allergic rhinitis (AR) and asthma preferentially have increased levels of cTfh cells that exhibit a Tfh2 cell phenotype (252). In patients with seasonal AR to ragweed pollen, activation of Tfh cells increases significantly during peak-season (253). It has been noted that the acute anaphylaxis response to peanut allergen is driven by IL-4⁺IL21⁺ Tfh cells and to a lesser extent by Th2 cells (254). Presence of Tfh cells but not Th2 cells is required for IgE production and for the development of an allergic response.

The increased levels of serum IgE is also a hallmark of atopic dermatitis (AD) (255–258). Peripheral Tfh cells in children with

AD have significantly increased levels of ICOS, PD-1, and IL-21 suggesting a highly activated phenotype. Furthermore, a strong positive correlation has been detected between the numbers of IL-21⁺ Tfh-like cells, activated memory B cell pool, and disease severity (259, 260). Interestingly, patients with asthma also display elevated levels of PD-1⁺ICOS⁺ Tfh2 cells and the ratio of Tfh2:Tfh1 cells positively correlates with the total IgE levels in the blood (46). In correlation with the data on human samples, mRNA and protein expression levels of CXCR5, ICOS, ICOSL, and IL-21 were also elevated in mouse models of asthma. Our recent findings also identify an important role of the TF Batf toward the generation of IL-4-expressing Tfh cells rather than Th2 and to their pro-allergic function (120). We further demonstrated that the IL-4-STAT6 signaling contributes to the Batf induction in Tfh cells and the Batf/IRF4 complex along with Stat3 and Stat6 aids IL-4 production in Tfh cells. Recent studies have also shown that Tfh cells can sense specific microenvironmental conditions and differentiate into Th2 cells after repeat exposure with house dust mite (HDM) (261). At the initial stage of disease, Tfh cells are preferentially differentiated, but a second exposure leads to the Tfh switch into Th2 cells which migrate to the lung and produce an inflammatory response. These results suggest that targeting Tfh cells may be a good therapeutic strategy to prevent Th2-cell-mediated immunity to HDM (261).

Primary Immunodeficiency

There are several immunodeficiencies that directly affect the development and functions of Tfh cells and as a result alter B cell-dependent responses. XLP is a primary immunodeficiency caused by mutations in SH2D1A (encoding for SAP, signaling lymphocytic activation molecule-associated protein). A SAP deficiency does not impact CD4⁺ T cell development, but compromises Tfh cell differentiation (262). In line with this observation, XLP patients exhibited significant defects in GC formation, reductions in memory B cell responses, hypogammaglobulinemia, and impaired antigen-specific antibody responses (263). Mutations in CD40L, ICOS, and STAT3 also cause reduced number of CD4⁺CXCR5⁺ cells, defective GC formation, and impaired humoral immune responses. A recent study also highlighted the importance of the IL-12/IL-12R axis as patients with mutations in IL-12R β 1 demonstrate fewer circulating memory Tfh, memory B cells, and defective GCs compared with control subject (264).

HIV and SIV Pathologies

Proper activation of Tfh cells and their interactions with GC B cells are essential for an effective humoral immune response and the extermination of pathogenic human and simian immunodeficiency viruses (HIV and SIV, respectively). Paradoxically, HIV-infected individuals (265) and monkeys infected with SIV (266) display a significantly higher frequency of ICOS⁺ Tfh, PD-1⁺ Tfh, and ICOS⁺PD-1⁺ Tfh cells among total CD4⁺ T cells compared with non-infected controls. Furthermore, Tfh cell frequency is significantly higher in non-treated HIV⁺ patients compared with HIV⁺ patients treated with combination antiretroviral therapy, suggesting that an HIV viral persistence contributes to Tfh cell expansion (265–267). Interestingly, Tfh cells

in humans and macaques show preferential infection with HIV and SIV, respectively (200, 267, 268), likely *via* the chemokine receptor CCR5 as CCR5 is expressed on a precursor subset of Tfh cells and may potentially serve as a co-receptor for HIV (269). GC Tfh cells have been implicated in HIV persistence by supporting viral replication during treated infection and serve as an important cellular reservoir of HIV-1 DNA (270, 271). One explanation is that cytotoxic CD8⁺ T cells are CXCR5 negative and thus unable to migrate to follicles and target HIV-infected GC Tfh cells (200, 272, 273). In line with an increased number of Tfh cells, hypergammaglobulinemia is detected in HIV⁺ patients (274), but these antibodies are ineffective (265, 273, 275–278). Studies have proposed that PD-1 triggering by PD-L1 on GC B cells is a mechanism for the abnormal Tfh functions and defective B cell help (277). In addition, due to the increased number of Tfh cells, the exacerbated interactions of Tfh and GC B cells may lower the threshold for B cell selection resulting in the selection of B cells with low affinities (223). Thus, the relationship between viral entrance into Tfh cells, number and functions of Tfh cells, and B cell activation/maturation is complex and requires further investigations.

Chronic Infections

Lymphocytic choriomeningitis virus persistence results in extended TCR engagement and an IL-6-driven shift from a Th1-induced response toward a Tfh response (279, 280). In addition to studies that identified a key role of Tfh cells in HIV and SIV infections, studies with the LCMV disease model further highlight the importance of timely expansion and sustained functions of Tfh cells in the orchestration of a proper humoral response for control of persistent viral infection (279, 280). Moreover, annual influenza virus studies determined an increase in the number and activation levels of cTfh cells. These studies not only show that an increased cTfh cell frequency contributes to circulating plasmablast responses and infection clearance but can also be used as a marker to monitor the efficacy of influenza vaccination (281, 282). In other infections such as chronic hepatitis B virus patients have an increase in circulating regulatory Tfh cells (283). Interestingly, long-term disruptions of proper T cell-dependent Ab production have been detected in the case of parasitic infections such as *Leishmania* (284), *Litomosoides sigmodontis* (285), and *Plasmodium* infection (286) suggesting the negative regulation of the number and functions of Tfh cells under these conditions. To date, the precise mechanisms that regulate differentiations and functions of Tfh and Tfr cells in different infection settings remain to be determined. Particularly, further studies are required to assess the mechanisms governing Tfh cell development, persistence, and function at the different stages of various infection diseases.

Cancer

Accumulating evidence suggests that Tfh cells are involved in peripheral T cell and B cell-associated tumors due to their high impact on growth and survival of different leukocyte subsets. Angioimmunoblastic T cell lymphoma (AITL) is an aggressive tumor and isolated neoplastic T cells express CXCL13, ICOS, CD154, CD40L, and NFATC1 (287, 288), making these T cells

similar to Tfh cells (289). The expression of mutated RhoA G17V induces Tfh cell differentiation/activation; increased proliferation associated with ICOS upregulation, elevated PI3K, and mitogen-activated protein signaling (290). Loss-of-function mutations in epigenetic regulators such as TET2 and DNMT3A are frequent events in the pathogenesis of AITL. Interestingly, RhoA G17V expression accompanied with TET2 loss results in AITL development in mice. It is worth to note that altered RhoA GTPase activity has been linked with autoimmunity and studies in AITL further highlight a role of RhoA in shaping Tfh cell phenotype and response.

Like AITL, in follicular T cell lymphomas, infiltrating T cells resemble a phenotype of Tfh-like cells and express IL-4, TNF- α , IFN- γ , LT- α , CCL17, and CCL22 chemokines that play a role in the regulation of Treg and Th2 cell migration and modulate the activity of GC B cells within follicles as well. Not only Tfh-like cells but also Foxp3⁺ Tfr cells are found within neoplastic follicles and the number of Tfr cells is elevated during progression lymphomagenesis. Thus, there is a complex T cell response that is regulated by a delicate balance of CD4⁺, Tregs, and Tfh subsets. Probably one of the strongest predictors of survival would be the location pattern for T cell subsets, as accumulation within the follicles is linked with poor survival compared with a distribution pattern outside the follicle (291). To date, there is limited understanding in the functions of Tfh and Tfr subsets in lymphomagenesis and more detailed research in animal models and human samples will help to dissect the complex role of Tfh cells in cancer.

Unexpectedly, Tfh cells have protective roles in nonlymphoid tumors. Higher levels of Tfh cell infiltrates and their ability to organize tertiary lymphoid structures within tumors has been associated with increased survival and reduced immunosuppression which strongly correlate with an increased survival in breast cancer (292). Evidence suggest that IL-21 and CXCL13 may play a key role in the protective functions of Tfh cells *via* the modulation of local leukocyte recruitment. Infiltrating Tfh cells have also been reported in chronic lymphocytic leukemia, non-small cell lung cancer, osteosarcoma, and colorectal cancer (214, 293–297), where they positively correlated with patient survival (293). So far, very little is known about how Tfh cells impact the immune response involved in the suppression of tumor initiation and progression and further studies will be important for a better understanding of Tfh-related pathologies.

CONCLUSION

Since the identification of Tfh cells and the discovery of Bcl6 as a critical factor in their generation, there has been substantial progress made in understanding the molecular and cellular requirement for the development and function of mouse and human Tfh cells. To date, multiple cell-extrinsic and -intrinsic factors (costimulatory molecules, cytokines signaling, and transcriptional factors) have been determined to positively or negatively contribute to Tfh cell development. However, still many questions remain to be answered: (i) What is the exact composition and hierarchy of these factors and what are their

stage-specific requirements? (ii) Is Bcl6 required complete Tfh cell commitment? (iii) How Tfh-specific transcriptional factors impact epigenetic mechanisms governing Tfh cell generation? (iv) Which factors contribute to Tfh cell maintenance and memory formation? (v) What are the appropriate Tfh-specific target(s) for therapy in Tfh mediated autoimmune disorders and cancer?

T follicular helper cells mainly localize in secondary lymphoid organs and circulate in the blood and are beginning to emerge as crucial players in maintaining a healthy balance between protective and pathogenic immunity (Figure 2). However, due to the localization of Tfh cells in secondary lymphoid tissues, the study of human Tfh cell heterogeneity and function in normal and disease settings has been difficult. Further analysis and comparison of human circulating counterparts to tissue-resident Tfh cells in various disease settings is critical, since it will help to reveal the level of Tfh cell heterogeneity at various stages of diseases as well as will determine whether the disease-associated alterations in Tfh cells are the main cause or result of disorders. Thus, in view of an emerging role of Tfh cells in various disease settings, we believe that current progress and further understanding of the heterogeneity and regulation

of tissue-specific and cTfh cells in health and disease will lead to improved vaccine designs, better management of major autoimmune, inflammatory disorders, and cancer, and can be utilized as a novel prognostic biomarker for the identification of lymphoid and solid tumors.

AUTHOR CONTRIBUTIONS

All authors listed contributed significantly toward the preparation of the manuscript. LQ, TW, SB, EG, and RN wrote the manuscript. AS helped in writing, extensive editing, and revising the manuscript. HZ provided critical comments on the manuscript.

ACKNOWLEDGMENTS

This work is supported by NIH research grants (RN) (R03CA219760, R21AI120012, and R56AI125269), Institutional Research Grant (RN), and R01 HL139000 (EG and RN). RN and LQ are recipients of China Scholarship Council Fellowship (201706070066).

REFERENCES

- Vinuesa CG, Linterman MA, Yu D, MacLennan IC. Follicular helper T cells. *Annu Rev Immunol* (2016) 34:335–68. doi:10.1146/annurev-immunol-041015-055605
- Wali S, Sahoo A, Puri S, Alekseev A, Nurieva R. Insights into the development and regulation of T follicular helper cells. *Cytokine* (2016) 87:9–19. doi:10.1016/j.cyt.2016.06.010
- Crotty S. Follicular helper CD4 T cells (TFH). *Annu Rev Immunol* (2011) 29:621–63. doi:10.1146/annurev-immunol-031210-101400
- Mesquita D Jr, Cruvinel WM, Resende LS, Mesquita FV, Silva NP, Camara NO, et al. Follicular helper T cell in immunity and autoimmunity. *Braz J Med Biol Res* (2016) 49:e5209. doi:10.1590/1414-431X20165209
- Karnowski A, Chevrier S, Belz GT, Mount A, Emslie D, D'Costa K, et al. B and T cells collaborate in antiviral responses via IL-6, IL-21, and transcriptional activator and coactivator, Oct2 and OBF-1. *J Exp Med* (2012) 209:2049–64. doi:10.1084/jem.20111504
- Nurieva RI, Chung Y, Hwang D, Yang XO, Kang HS, Ma L, et al. Generation of T follicular helper cells is mediated by interleukin-21 but independent of T helper 1, 2, or 17 cell lineages. *Immunity* (2008) 29:138–49. doi:10.1016/j.immuni.2008.05.009
- Nurieva RI, Chung Y, Martinez GJ, Yang XO, Tanaka S, Matskevitch TD, et al. Bcl6 mediates the development of T follicular helper cells. *Science* (2009) 325:1001–5. doi:10.1126/science.1176676
- Schmitt N, Liu Y, Bentebibel SE, Munagala I, Boudry L, Venuprasad K, et al. The cytokine TGF-beta co-opts signaling via STAT3-STAT4 to promote the differentiation of human TFH cells. *Nat Immunol* (2014) 15:856–65. doi:10.1038/ni.2947
- Locci M, Wu JE, Arumemi F, Mikulski Z, Dahlberg C, Miller AT, et al. Activin A programs the differentiation of human TFH cells. *Nat Immunol* (2016) 17:976–84. doi:10.1038/ni.3494
- Ballesteros-Tato A, Leon B, Graf BA, Moquin A, Adams PS, Lund FE, et al. Interleukin-2 inhibits germinal center formation by limiting T follicular helper cell differentiation. *Immunity* (2012) 36:847–56. doi:10.1016/j.immuni.2012.02.012
- McDonald PW, Read KA, Baker CE, Anderson AE, Powell MD, Ballesteros-Tato A, et al. IL-7 signalling represses Bcl-6 and the TFH gene program. *Nat Commun* (2016) 7:10285. doi:10.1038/ncomms10285
- Lages CS, Lewkowich I, Sproles A, Wills-Karp M, Choungnet C. Partial restoration of T-cell function in aged mice by in vitro blockade of the PD-1/PD-L1 pathway. *Aging Cell* (2010) 9:785–98. doi:10.1111/j.1474-9726.2010.00611.x
- Sage PT, Paterson AM, Lovitch SB, Sharpe AH. The coinhibitory receptor CTLA-4 controls B cell responses by modulating T follicular helper, T follicular regulatory, and T regulatory cells. *Immunity* (2014) 41:1026–39. doi:10.1016/j.immuni.2014.12.005
- Nurieva RI, Podd A, Chen Y, Alekseev AM, Yu M, Qi X, et al. STAT5 protein negatively regulates T follicular helper (Tfh) cell generation and function. *J Biol Chem* (2012) 287:11234–9. doi:10.1074/jbc.M111.324046
- Stone EL, Pepper M, Katayama CD, Kerdiles YM, Lai CY, Emslie E, et al. ICOS coreceptor signaling inactivates the transcription factor FOXO1 to promote Tfh cell differentiation. *Immunity* (2015) 42:239–51. doi:10.1016/j.immuni.2015.01.017
- Weinreich MA, Takada K, Skon C, Reiner SL, Jameson SC, Hogquist KA. KLF2 transcription-factor deficiency in T cells results in unrestrained cytokine production and upregulation of bystander chemokine receptors. *Immunity* (2009) 31:122–30. doi:10.1016/j.immuni.2009.05.011
- Kim CH, Rott LS, Clark-Lewis I, Campbell DJ, Wu L, Butcher EC. Subspecialization of CXCR5+ T cells: B helper activity is focused in a germinal center-localized subset of CXCR5+ T cells. *J Exp Med* (2001) 193:1373–81. doi:10.1084/jem.193.12.1373
- Chtanova T, Tangye SG, Newton R, Frank N, Hodge MR, Rolph MS, et al. T follicular helper cells express a distinctive transcriptional profile, reflecting their role as non-Th1/Th2 effector cells that provide help for B cells. *J Immunol* (2004) 173:68–78. doi:10.4049/jimmunol.173.1.68
- Breitfeld D, Ohl L, Kremmer E, Ellwart J, Sallusto F, Lipp M, et al. Follicular B helper T cells express CXC chemokine receptor 5, localize to B cell follicles, and support immunoglobulin production. *J Exp Med* (2000) 192:1545–52. doi:10.1084/jem.192.11.1545
- Schaerli P, Willmann K, Lang AB, Lipp M, Loetscher P, Moser B. CXC chemokine receptor 5 expression defines follicular homing T cells with B cell helper function. *J Exp Med* (2000) 192:1553–62. doi:10.1084/jem.192.11.1553
- Ansel KM, McHeyzer-Williams LJ, Ngo VN, McHeyzer-Williams MG, Cyster JG. In vivo-activated CD4 T cells upregulate CXC chemokine receptor 5 and reprogram their response to lymphoid chemokines. *J Exp Med* (1999) 190:1123–34. doi:10.1084/jem.190.8.1123
- Forster R, Mattis AE, Kremmer E, Wolf E, Brem G, Lipp M. A putative chemokine receptor, BLR1, directs B cell migration to defined lymphoid organs and specific anatomic compartments of the spleen. *Cell* (1996) 87:1037–47. doi:10.1016/S0092-8674(00)81798-5

23. Ma CS, Deenick EK, Batten M, Tangye SG. The origins, function, and regulation of T follicular helper cells. *J Exp Med* (2012) 209:1241–53. doi:10.1084/jem.20120994
24. Gunn MD, Ngo VN, Ansel KM, Ekland EH, Cyster JG, Williams LT. A B-cell-homing chemokine made in lymphoid follicles activates Burkitt's lymphoma receptor-1. *Nature* (1998) 391:799–803. doi:10.1038/35876
25. Kim CH, Lim HW, Kim JR, Rott L, Hillsamer P, Butcher EC. Unique gene expression program of human germinal center T helper cells. *Blood* (2004) 104:1952–60. doi:10.1182/blood-2004-03-1206
26. Johnston RJ, Poholek AC, DiToro D, Yusuf I, Eto D, Barnett B, et al. Bcl6 and Blimp-1 are reciprocal and antagonistic regulators of T follicular helper cell differentiation. *Science* (2009) 325:1006–10. doi:10.1126/science.1175870
27. Yu D, Rao S, Tsai LM, Lee SK, He Y, Sutcliffe EL, et al. The transcriptional repressor Bcl-6 directs T follicular helper cell lineage commitment. *Immunity* (2009) 31:457–68. doi:10.1016/j.immuni.2009.07.002
28. Ueno H, Banachereau J, Vinuesa CG. Pathophysiology of T follicular helper cells in humans and mice. *Nat Immunol* (2015) 16:142–52. doi:10.1038/ni.3054
29. Flynn S, Toellner KM, Raykundalia C, Goodall M, Lane P. CD4 T cell cytokine differentiation: the B cell activation molecule, OX40 ligand, instructs CD4 T cells to express interleukin 4 and upregulates expression of the chemokine receptor, Bcl-1. *J Exp Med* (1998) 188:297–304. doi:10.1084/jem.188.2.297
30. Kerfoot SM, Yaari G, Patel JR, Johnson KL, Gonzalez DG, Kleinstein SH, et al. Germinal center B cell and T follicular helper cell development initiates in the interfollicular zone. *Immunity* (2011) 34:947–60. doi:10.1016/j.immuni.2011.03.024
31. Kitano M, Moriyama S, Ando Y, Hikida M, Mori Y, Kurosaki T, et al. Bcl6 protein expression shapes pre-germinal center B cell dynamics and follicular helper T cell heterogeneity. *Immunity* (2011) 34:961–72. doi:10.1016/j.immuni.2011.03.025
32. Haynes NM, Allen CD, Lesley R, Ansel KM, Killeen N, Cyster JG. Role of CXCR5 and CCR7 in follicular Th cell positioning and appearance of a programmed cell death gene-1-high germinal center-associated subpopulation. *J Immunol* (2007) 179:5099–108. doi:10.4049/jimmunol.179.8.5099
33. Ramiscal RR, Vinuesa CG. T-cell subsets in the germinal center. *Immunol Rev* (2013) 252:146–55. doi:10.1111/imr.12031
34. Bentebibel SE, Schmitt N, Banachereau J, Ueno H. Human tonsil B-cell lymphoma 6 (BCL6)-expressing CD4+ T-cell subset specialized for B-cell help outside germinal centers. *Proc Natl Acad Sci U S A* (2011) 108:E488–97. doi:10.1073/pnas.1100898108
35. Nakayama S, Kanno Y, Takahashi H, Jankovic D, Lu KT, Johnson TA, et al. Early Th1 cell differentiation is marked by a Tfh cell-like transition. *Immunity* (2011) 35:919–31. doi:10.1016/j.immuni.2011.11.012
36. Bauquet AT, Jin H, Paterson AM, Mitsdoerffer M, Ho IC, Sharpe AH, et al. The costimulatory molecule ICOS regulates the expression of c-Maf and IL-21 in the development of follicular T helper cells and TH-17 cells. *Nat Immunol* (2009) 10:167–75. doi:10.1038/ni.1690
37. He J, Tsai LM, Leong YA, Hu X, Ma CS, Chevalier N, et al. Circulating precursor CCR7(lo)PD-1(hi) CXCR5(+) CD4(+) T cells indicate Tfh cell activity and promote antibody responses upon antigen reexposure. *Immunity* (2013) 39:770–81. doi:10.1016/j.immuni.2013.09.007
38. Morita R, Schmitt N, Bentebibel SE, Ranganathan R, Bourdery L, Zurawski G, et al. Human blood CXCR5(+)CD4(+) T cells are counterparts of T follicular cells and contain specific subsets that differentially support antibody secretion. *Immunity* (2011) 34:108–21. doi:10.1016/j.immuni.2010.12.012
39. Schmitt N, Bentebibel SE, Ueno H. Phenotype and functions of memory Tfh cells in human blood. *Trends Immunol* (2014) 35:436–42. doi:10.1016/j.it.2014.06.002
40. Asrir A, Aloulou M, Gador M, Peralas C, Fazilleau N. Interconnected subsets of memory follicular helper T cells have different effector functions. *Nat Commun* (2017) 8:847. doi:10.1038/s41467-017-00843-7
41. Scherm MG, Ott VB, Daniel C. Follicular helper T cells in autoimmunity. *Curr Diab Rep* (2016) 16:75. doi:10.1007/s11892-016-0770-2
42. Schmitt N, Ueno H. Regulation of human helper T cell subset differentiation by cytokines. *Curr Opin Immunol* (2015) 34:130–6. doi:10.1016/j.coi.2015.03.007
43. Weinstein JS, Hernandez SG, Craft J. T cells that promote B-cell maturation in systemic autoimmunity. *Immunol Rev* (2012) 247:160–71. doi:10.1111/j.1600-065X.2012.01122.x
44. Jogdand GM, Mohanty S, Devadas S. Regulators of Tfh cell differentiation. *Front Immunol* (2016) 7:520. doi:10.3389/fimmu.2016.00520
45. Yang JH, Zhang J, Cai Q, Zhao DB, Wang J, Guo PE, et al. Expression and function of inducible costimulator on peripheral blood T cells in patients with systemic lupus erythematosus. *Rheumatology (Oxford)* (2005) 44:1245–54. doi:10.1093/rheumatology/keh724
46. Gong F, Qian C, Zhu H, Zhu J, Pan Y, Dong Q, et al. Circulating follicular T-helper cell subset distribution in patients with asthma. *Allergy Asthma Proc* (2016) 37:154–61. doi:10.2500/aap.2016.37.3982
47. Weber JP, Fuhrmann F, Feist RK, Lahmann A, Al Baz MS, Gentz LJ, et al. ICOS maintains the T follicular helper cell phenotype by down-regulating Kruppel-like factor 2. *J Exp Med* (2015) 212:217–33. doi:10.1084/jem.20141432
48. Alegre ML, Frauwirth KA, Thompson CB. T-cell regulation by CD28 and CTLA-4. *Nat Rev Immunol* (2001) 1:220–8. doi:10.1038/35105024
49. Peach RJ, Bajorath J, Brady W, Leytze G, Greene J, Naemura J, et al. Complementarity determining region 1 (CDR1)- and CDR3-analogous regions in CTLA-4 and CD28 determine the binding to B7-1. *J Exp Med* (1994) 180(6):2049–58. doi:10.1084/jem.180.6.2049
50. Bluestone J. New perspectives of CD28-B7-mediated T cell costimulation. *Immunity* (1995) 2(6):555–9. doi:10.1016/1074-7613(95)90000-4
51. Salek-Ardakani S, Choi YS, Rafii-El-Idrissi Benhnia M, Flynn R, Arens R, Shoenberger S, et al. B cell-specific expression of B7-2 is required for follicular Th cell function in response to vaccinia virus. *J Immunol* (2011) 186:5294–303. doi:10.4049/jimmunol.1100406
52. Wang CJ, Heuts F, Ovcinnikovs V, Wardzinski L, Bowers C, Schmidt EM, et al. CTLA-4 controls follicular helper T-cell differentiation by regulating the strength of CD28 engagement. *Proc Natl Acad Sci U S A* (2015) 112:524–9. doi:10.1073/pnas.1414576112
53. Linterman MA, Denton AE, Divekar DP, Zvetkova I, Kane L, Ferreira C, et al. CD28 expression is required after T cell priming for helper T cell responses and protective immunity to infection. *Elife* (2014) 3:e03180. doi:10.7554/eLife.03180
54. Leavenworth JW, Verbinnen B, Yin J, Huang H, Cantor H. A p85alpha-osteopontin axis couples the receptor ICOS to sustained Bcl-6 expression by follicular helper and regulatory T cells. *Nat Immunol* (2015) 16:96–106. doi:10.1038/ni.3050
55. Chen S, Cai C, Li Z, Liu G, Wang Y, Blonska M, et al. Dissection of SAP-dependent and SAP-independent SLAM family signaling in NKT cell development and humoral immunity. *J Exp Med* (2017) 214:475–89. doi:10.1084/jem.20161312
56. Ma CS, Deenick EK. The role of SAP and SLAM family molecules in the humoral immune response. *Ann N Y Acad Sci* (2011) 1217:32–44. doi:10.1111/j.1749-6632.2010.05824.x
57. Cannons JL, Tangye SG, Schwartzberg PL. SLAM family receptors and SAP adaptors in immunity. *Annu Rev Immunol* (2011) 29:665–705. doi:10.1146/annurev-immunol-030409-101302
58. Cannons JL, Qi H, Lu KT, Dutta M, Gomez-Rodriguez J, Cheng J, et al. Optimal germinal center responses require a multistage T cell:B cell adhesion process involving integrins, SLAM-associated protein, and CD84. *Immunity* (2010) 32:253–65. doi:10.1016/j.immuni.2010.01.010
59. Kageyama R, Cannons JL, Zhao F, Yusuf I, Lao C, Locci M, et al. The receptor Ly108 functions as a SAP adaptor-dependent on-off switch for T cell help to B cells and NKT cell development. *Immunity* (2012) 36:986–1002. doi:10.1016/j.immuni.2012.05.016
60. Yusuf I, Kageyama R, Monticelli L, Johnston RJ, Ditoro D, Hansen K, et al. Germinal center T follicular helper cell IL-4 production is dependent on signaling lymphocytic activation molecule receptor (CD150). *J Immunol* (2010) 185:190–202. doi:10.4049/jimmunol.0903505
61. Crotty S, Kersh EN, Cannons J, Schwartzberg PL, Ahmed R. SAP is required for generating long-term humoral immunity. *Nature* (2003) 421:282–7. doi:10.1038/nature01318
62. Kroenke MA, Eto D, Locci M, Cho M, Davidson T, Haddad EK, et al. Bcl6 and Maf cooperate to instruct human follicular helper CD4 T cell differentiation. *J Immunol* (2012) 188:3734–44. doi:10.4049/jimmunol.1103246
63. Hu J, Havenar-Daughton C, Crotty S. Modulation of SAP dependent T:B cell interactions as a strategy to improve vaccination. *Curr Opin Virol* (2013) 3:363–70. doi:10.1016/j.coviro.2013.05.015

64. Cannons JL, Yu LJ, Jankovic D, Crotty S, Horai R, Kirby M, et al. SAP regulates T cell-mediated help for humoral immunity by a mechanism distinct from cytokine regulation. *J Exp Med* (2006) 203:1551–65. doi:10.1084/jem.20052097
65. Qi H, Cannons JL, Klauschen F, Schwartzberg PL, Germain RN. SAP-controlled T-B cell interactions underlie germinal centre formation. *Nature* (2008) 455:764–9. doi:10.1038/nature07345
66. Latour S, Roncagalli R, Chen R, Bakinowski M, Shi X, Schwartzberg PL, et al. Binding of SAP SH2 domain to FynT SH3 domain reveals a novel mechanism of receptor signalling in immune regulation. *Nat Cell Biol* (2003) 5:149–54. doi:10.1038/ncb919
67. Cannons JL, Wu JZ, Gomez-Rodriguez J, Zhang J, Dong B, Liu Y, et al. Biochemical and genetic evidence for a SAP-PKC-theta interaction contributing to IL-4 regulation. *J Immunol* (2010) 185:2819–27. doi:10.4049/jimmunol.0902182
68. Zhao F, Cannons JL, Dutta M, Griffiths GM, Schwartzberg PL. Positive and negative signaling through SLAM receptors regulate synapse organization and thresholds of cytotoxicity. *Immunity* (2012) 36:1003–16. doi:10.1016/j.immuni.2012.05.017
69. Nocentini G, Giunchi L, Ronchetti S, Krausz LT, Bartoli A, Moraca R, et al. A new member of the tumor necrosis factor/nerve growth factor receptor family inhibits T cell receptor-induced apoptosis. *Proc Natl Acad Sci U S A* (1997) 94:6216–21. doi:10.1073/pnas.94.12.6216
70. Knee DA, Hewes B, Brogdon JL. Rationale for anti-GITR cancer immunotherapy. *Eur J Cancer* (2016) 67:1–10. doi:10.1016/j.ejca.2016.06.028
71. Stephens GL, McHugh RS, Whitters MJ, Young DA, Luxenberg D, Carreno BM, et al. Engagement of glucocorticoid-induced TNFR family-related receptor on effector T cells by its ligand mediates resistance to suppression by CD4+CD25+ T cells. *J Immunol* (2004) 173:5008–20. doi:10.4049/jimmunol.173.8.5008
72. Butcher MJ, Filipowicz AR, Waseem TC, McGary CM, Crow KJ, Magilnick N, et al. Atherosclerosis-driven Treg plasticity results in formation of a dysfunctional subset of plastic IFN γ + Th1/Tregs. *Circ Res* (2016) 119:1190–203. doi:10.1161/CIRCRESAHA.116.309764
73. Clouthier DL, Zhou AC, Wortzman ME, Luft O, Levy GA, Watts TH. GITR intrinsically sustains early type 1 and late follicular helper CD4 T cell accumulation to control a chronic viral infection. *PLoS Pathog* (2015) 11:e1004517. doi:10.1371/journal.ppat.1004517
74. Good-Jacobson KL, Szumilas CG, Chen L, Sharpe AH, Tomayko MM, Shlomchik MJ. PD-1 regulates germinal center B cell survival and the formation and affinity of long-lived plasma cells. *Nat Immunol* (2010) 11:535–42. doi:10.1038/ni.1877
75. Chung Y, Tanaka S, Chu F, Nurieva RI, Martinez GJ, Rawal S, et al. Follicular regulatory T cells expressing Foxp3 and Bcl-6 suppress germinal center reactions. *Nat Med* (2011) 17:983–8. doi:10.1038/nm.2426
76. Linterman MA, Pierson W, Lee SK, Kallies A, Kawamoto S, Rayner TF, et al. Foxp3+ follicular regulatory T cells control the germinal center response. *Nat Med* (2011) 17:975–82. doi:10.1038/nm.2425
77. Sage PT, Francisco LM, Carman CV, Sharpe AH. The receptor PD-1 controls follicular regulatory T cells in the lymph nodes and blood. *Nat Immunol* (2013) 14:152–61. doi:10.1038/ni.2496
78. Sage PT, Sharpe AH. T follicular regulatory cells. *Immunol Rev* (2016) 271:246–59. doi:10.1111/immr.12411
79. Francisco LM, Sage PT, Sharpe AH. The PD-1 pathway in tolerance and autoimmunity. *Immunol Rev* (2010) 236:219–42. doi:10.1111/j.1600-065X.2010.00923.x
80. Keir ME, Butte MJ, Freeman GJ, Sharpe AH. PD-1 and its ligands in tolerance and immunity. *Annu Rev Immunol* (2008) 26:677–704. doi:10.1146/annurev.immunol.26.021607.090331
81. Hamel KM, Cao Y, Wang Y, Rodeghero R, Kobezda T, Chen L, et al. B7-H1 expression on non-B and non-T cells promotes distinct effects on T- and B-cell responses in autoimmune arthritis. *Eur J Immunol* (2010) 40:3117–27. doi:10.1002/eji.201040690
82. Kawamoto S, Tran TH, Maruya M, Suzuki K, Doi Y, Tsutsui Y, et al. The inhibitory receptor PD-1 regulates IgA selection and bacterial composition in the gut. *Science* (2012) 336:485–9. doi:10.1126/science.1217718
83. Hams E, McCarron MJ, Amu S, Yagita H, Azuma M, Chen L, et al. Blockade of B7-H1 (programmed death ligand 1) enhances humoral immunity by positively regulating the generation of T follicular helper cells. *J Immunol* (2011) 186:5648–55. doi:10.4049/jimmunol.1003161
84. Velu V, Titanji K, Zhu B, Husain S, Pladevega A, Lai L, et al. Enhancing SIV-specific immunity in vivo by PD-1 blockade. *Nature* (2009) 458:206–10. doi:10.1038/nature07662
85. Butler NS, Moebius J, Pewe LL, Traore B, Doumbo OK, Tygrett LT, et al. Therapeutic blockade of PD-L1 and LAG-3 rapidly clears established blood-stage Plasmodium infection. *Nat Immunol* (2011) 13:188–95. doi:10.1038/ni.2180
86. Sage PT, Tan CL, Freeman GJ, Haigis M, Sharpe AH. Defective TFH cell function and increased TFR cells contribute to defective antibody production in aging. *Cell Rep* (2015) 12:163–71. doi:10.1016/j.celrep.2015.06.015
87. Badell IR, La Muraglia GM II, Liu D, Wagener ME, Ding G, Ford ML. Selective CD28 blockade results in superior inhibition of donor-specific T follicular helper cell and antibody responses relative to CTLA4-Ig. *Am J Transplant* (2018) 18:89–101. doi:10.1111/ajt.14400
88. Walunas TL, Lenschow DJ, Bakker CY, Linsley PS, Freeman GJ, Green JM, et al. CTLA-4 can function as a negative regulator of T cell activation. *Immunity* (1994) 1:405–13. doi:10.1016/1074-7613(94)90071-X
89. Krummel MF. CD28 and CTLA-4 have opposing effects on the response of T cells to stimulation. *J Exp Med* (1995) 182:459–65. doi:10.1084/jem.182.2.459
90. Tivol EA, Borriello F, Schweitzer AN, Lynch WP, Bluestone JA, Sharpe AH. Loss of CTLA-4 leads to massive lymphoproliferation and fatal multiorgan tissue destruction, revealing a critical negative regulatory role of CTLA-4. *Immunity* (1995) 3:541–7. doi:10.1016/1074-7613(95)90125-6
91. Perez VL, Van Parijs L, Biuckians A, Zheng XX, Strom TB, Abbas AK. Induction of peripheral T cell tolerance in vivo requires CTLA-4 engagement. *Immunity* (1997) 6:411–7. doi:10.1016/S1074-7613(00)80284-8
92. Leach DR, Krummel MF, Allison JP. Enhancement of antitumor immunity by CTLA-4 blockade. *Science* (1996) 271:1734–6. doi:10.1126/science.271.5256.1734
93. Walker LS. Treg and CTLA-4: two intertwining pathways to immune tolerance. *J Autoimmun* (2013) 45:49–57. doi:10.1016/j.jaut.2013.06.006
94. Tivol EA, Boyd SD, McKeon S, Borriello F, Nickerson P, Strom TB, et al. CTLA4Ig prevents lymphoproliferation and fatal multiorgan tissue destruction in CTLA-4-deficient mice. *J Immunol* (1997) 158:5091–4.
95. Tang Q, Boden EK, Henriksen KJ, Bour-Jordan H, Bi M, Bluestone JA. Distinct roles of CTLA-4 and TGF- β in CD4+CD25+ regulatory T cell function. *Eur J Immunol* (2004) 34:2996–3005. doi:10.1002/eji.200425143
96. Wing K, Onishi Y, Prieto-Martin P, Yamaguchi T, Miyara M, Fehervari Z, et al. CTLA-4 control over Foxp3+ regulatory T cell function. *Science* (2008) 322:271–5. doi:10.1126/science.1160062
97. Wing JB, Ise W, Kurosaki T, Sakaguchi S. Regulatory T cells control antigen-specific expansion of Tfh cell number and humoral immune responses via the coreceptor CTLA-4. *Immunity* (2014) 41:1013–25. doi:10.1016/j.immuni.2014.12.006
98. Cheung TC, Humphreys IR, Potter KG, Norris PS, Shumway HM, Tran BR, et al. Evolutionarily divergent herpesviruses modulate T cell activation by targeting the herpesvirus entry mediator cosignaling pathway. *Proc Natl Acad Sci U S A* (2005) 102:13218–23. doi:10.1073/pnas.0506172102
99. del Rio ML, Jones ND, Buhler L, Norris P, Shintani Y, Ware CF, et al. Selective blockade of herpesvirus entry mediator-B and T lymphocyte attenuator pathway ameliorates acute graft-versus-host reaction. *J Immunol* (2012) 188:4885–96. doi:10.4049/jimmunol.1103698
100. del Rio ML, Kaye J, Rodriguez-Barbosa JI. Detection of protein on BTLA on lymphoid and myeloid cells of C57BL/6 and BALB/c BTLA allelic variants. *Immunobiology* (2010) 215:570–8. doi:10.1016/j.imbio.2009.09.008
101. Oya Y, Watanabe N, Owada T, Oki M, Hirose K, Suto A, et al. Development of autoimmune hepatitis-like disease and production of autoantibodies to nuclear antigens in mice lacking B and T lymphocyte attenuator. *Arthritis Rheum* (2008) 58:2498–510. doi:10.1002/art.23674
102. Kashiwakuma D, Suto A, Hiramatsu Y, Ikeda K, Takatori H, Suzuki K, et al. B and T lymphocyte attenuator suppresses IL-21 production from follicular Th cells and subsequent humoral immune responses. *J Immunol* (2010) 185:2730–6. doi:10.4049/jimmunol.0903839

103. Eto D, Lao C, DiToro D, Barnett B, Escobar TC, Kageyama R, et al. IL-21 and IL-6 are critical for different aspects of B cell immunity and redundantly induce optimal follicular helper CD4 T cell (Tfh) differentiation. *PLoS One* (2011) 6:e17739. doi:10.1371/journal.pone.0017739
104. Batten M, Ramamoorthi N, Kljavin NM, Ma CS, Cox JH, Dengler HS, et al. IL-27 supports germinal center function by enhancing IL-21 production and the function of T follicular helper cells. *J Exp Med* (2010) 207:2895–906. doi:10.1084/jem.20100064
105. Nurieva RI, Chung Y. Understanding the development and function of T follicular helper cells. *Cell Mol Immunol* (2010) 7:190–7. doi:10.1038/cmi.2010.24
106. Liu X, Nurieva RI, Dong C. Transcriptional regulation of follicular T-helper (Tfh) cells. *Immunol Rev* (2013) 252:139–45. doi:10.1111/imr.12040
107. Choi YS, Eto D, Yang JA, Lao C, Crotty S. Cutting edge: STAT1 is required for IL-6-mediated Bcl6 induction for early follicular helper cell differentiation. *J Immunol* (2013) 190:3049–53. doi:10.4049/jimmunol.1203032
108. Chavele KM, Merry E, Ehrenstein MR. Cutting edge: circulating plasmablasts induce the differentiation of human T follicular helper cells via IL-6 production. *J Immunol* (2015) 194:2482–5. doi:10.4049/jimmunol.1401190
109. Nurieva R, Yang XO, Martinez G, Zhang Y, Panopoulos AD, Ma L, et al. Essential autocrine regulation by IL-21 in the generation of inflammatory T cells. *Nature* (2007) 448:480–3. doi:10.1038/nature05969
110. Vogelzang A, McGuire HM, Yu D, Sprent J, Mackay CR, King C. A fundamental role for interleukin-21 in the generation of T follicular helper cells. *Immunity* (2008) 29:127–37. doi:10.1016/j.immuni.2008.06.001
111. Gringhuis SI, Kaptein TM, Wevers BA, van der Vlist M, Klaver EJ, van Die I, et al. Fucose-based PAMPs prime dendritic cells for follicular T helper cell polarization via DC-SIGN-dependent IL-27 production. *Nat Commun* (2014) 5:5074. doi:10.1038/ncomms6074
112. Owaki T, Asakawa M, Kamiya S, Takeda K, Fukai F, Mizuguchi J, et al. IL-27 suppresses CD28-mediated [correction of medicated] IL-2 production through suppressor of cytokine signaling 3. *J Immunol* (2006) 176:2773–80. doi:10.4049/jimmunol.176.5.2773
113. Peng X, Remacle JE, Kasran A, Huylebroeck D, Ceuppens JL. IL-12 up-regulates CD40 ligand (CD154) expression on human T cells. *J Immunol* (1998) 160:1166–72.
114. McCarron MJ, Marie JC. TGF-beta prevents T follicular helper cell accumulation and B cell autoreactivity. *J Clin Invest* (2014) 124:4375–86. doi:10.1172/JCI76179
115. Choi YS, Kageyama R, Eto D, Escobar TC, Johnston RJ, Monticelli L, et al. ICOS receptor instructs T follicular helper cell versus effector cell differentiation via induction of the transcriptional repressor Bcl6. *Immunity* (2011) 34:932–46. doi:10.1016/j.immuni.2011.03.023
116. Pepper M, Pagan AJ, Igyarto BZ, Taylor JJ, Jenkins MK. Opposing signals from the Bcl6 transcription factor and the interleukin-2 receptor generate T helper 1 central and effector memory cells. *Immunity* (2011) 35:583–95. doi:10.1016/j.immuni.2011.09.009
117. Liao W, Lin JX, Wang L, Li P, Leonard WJ. Modulation of cytokine receptors by IL-2 broadly regulates differentiation into helper T cell lineages. *Nat Immunol* (2011) 12:551–9. doi:10.1038/ni.2030
118. Ray JP, Staron MM, Shyer JA, Ho PC, Marshall HD, Gray SM, et al. The interleukin-2-mTORC1 kinase axis defines the signaling, differentiation, and metabolism of T helper 1 and follicular B Helper T cells. *Immunity* (2015) 43:690–702. doi:10.1016/j.immuni.2015.08.017
119. Cai G, Nie X, Zhang W, Wu B, Lin J, Wang H, et al. A regulatory role for IL-10 receptor signaling in development and B cell help of T follicular helper cells in mice. *J Immunol* (2012) 189:1294–302. doi:10.4049/jimmunol.1102948
120. Sahoo A, Alekseev A, Tanaka K, Obertas L, Lerman B, Haymaker C, et al. Batf is important for IL-4 expression in T follicular helper cells. *Nat Commun* (2015) 6:7997. doi:10.1038/ncomms8997
121. Gong F, Su Q, Jiang D, Chen J, Pan Y, Huang X. High frequency of circulating follicular helper T cells in patients with bronchial asthma. *Clin Lab* (2014) 60:963–8. doi:10.7754/Clin.Lab.2013.130427
122. Crotty S, Johnston RJ, Schoenberger SP. Effectors and memories: Bcl-6 and Blimp-1 in T and B lymphocyte differentiation. *Nat Immunol* (2010) 11:114–20. doi:10.1038/ni.1837
123. Martins G, Calame K. Regulation and functions of Blimp-1 in T and B lymphocytes. *Annu Rev Immunol* (2008) 26:133–69. doi:10.1146/annurev.immunol.26.021607.090241
124. Hatzi K, Nance JP, Kroenke MA, Bothwell M, Haddad EK, Melnick A, et al. BCL6 orchestrates Tfh cell differentiation via multiple distinct mechanisms. *J Exp Med* (2015) 212:539–53. doi:10.1084/jem.20141380
125. Kusam S, Toney LM, Sato H, Dent AL. Inhibition of Th2 differentiation and GATA-3 expression by BCL-6. *J Immunol* (2003) 170:2435–41. doi:10.4049/jimmunol.170.5.2435
126. Liu X, Lu H, Chen T, Nallaparaju KC, Yan X, Tanaka S, et al. Genome-wide analysis identifies Bcl6-controlled regulatory networks during T follicular helper cell differentiation. *Cell Rep* (2016) 14:1735–47. doi:10.1016/j.celrep.2016.01.038
127. Andris F, Denanglaire S, Anciaux M, Hercor M, Hussein H, Leo O. The transcription factor c-Maf promotes the differentiation of follicular helper T cells. *Front Immunol* (2017) 8:480. doi:10.3389/fimmu.2017.00480
128. Schraml BU, Hildner K, Ise W, Lee WL, Smith WA, Solomon B, et al. The AP-1 transcription factor Batf controls T(H)17 differentiation. *Nature* (2009) 460:405–9. doi:10.1038/nature08114
129. Betz BC, Jordan-Williams KL, Wang C, Kang SG, Liao J, Logan MR, et al. Batf coordinates multiple aspects of B and T cell function required for normal antibody responses. *J Exp Med* (2010) 207:933–42. doi:10.1084/jem.20091548
130. Ise W, Kohyama M, Schraml BU, Zhang T, Schwer B, Basu U, et al. The transcription factor BATF controls the global regulators of class-switch recombination in both B cells and T cells. *Nat Immunol* (2011) 12:536–43. doi:10.1038/ni.2037
131. Brustle A, Heink S, Huber M, Rosenplanter C, Stadelmann C, Yu P, et al. The development of inflammatory T(H)-17 cells requires interferon-regulatory factor 4. *Nat Immunol* (2007) 8:958–66. doi:10.1038/ni1500
132. Huber M, Brustle A, Reinhard K, Guralnik A, Walter G, Mahiny A, et al. IRF4 is essential for IL-21-mediated induction, amplification, and stabilization of the Th17 phenotype. *Proc Natl Acad Sci U S A* (2008) 105:20846–51. doi:10.1073/pnas.0809077106
133. Lohoff M, Mittrucker HW, Prechtel S, Bischof S, Sommer F, Kock S, et al. Dysregulated T helper cell differentiation in the absence of interferon regulatory factor 4. *Proc Natl Acad Sci U S A* (2002) 99:11808–12. doi:10.1073/pnas.182425099
134. Rengarajan J, Mowen KA, McBride KD, Smith ED, Singh H, Glimcher LH. Interferon regulatory factor 4 (IRF4) interacts with NFATc2 to modulate interleukin 4 gene expression. *J Exp Med* (2002) 195:1003–12. doi:10.1084/jem.20011128
135. Zheng Y, Chaudhry A, Kas A, deRoos P, Kim JM, Chu TT, et al. Regulatory T-cell suppressor program co-opts transcription factor IRF4 to control T(H)2 responses. *Nature* (2009) 458:351–6. doi:10.1038/nature07674
136. Chen Q, Yang W, Gupta S, Biswas P, Smith P, Bhagat G, et al. IRF-4-binding protein inhibits interleukin-17 and interleukin-21 production by controlling the activity of IRF-4 transcription factor. *Immunity* (2008) 29:899–911. doi:10.1016/j.immuni.2008.10.011
137. Staudt V, Bothur E, Klein M, Lingnau K, Reuter S, Grebe N, et al. Interferon-regulatory factor 4 is essential for the developmental program of T helper 9 cells. *Immunity* (2010) 33:192–202. doi:10.1016/j.immuni.2010.07.014
138. Klein U, Casola S, Cattoretto G, Shen Q, Lia M, Mo T, et al. Transcription factor IRF4 controls plasma cell differentiation and class-switch recombination. *Nat Immunol* (2006) 7:773–82. doi:10.1038/ni1357
139. Sciammas R, Shaffer AL, Schatz JH, Zhao H, Staudt LM, Singh H. Graded expression of interferon regulatory factor-4 coordinates isotype switching with plasma cell differentiation. *Immunity* (2006) 25:225–36. doi:10.1016/j.immuni.2006.07.009
140. Bollig N, Brustle A, Kellner K, Ackermann W, Abass E, Raifer H, et al. Transcription factor IRF4 determines germinal center formation through follicular T-helper cell differentiation. *Proc Natl Acad Sci U S A* (2012) 109:8664–9. doi:10.1073/pnas.1205834109
141. Li P, Spolski R, Liao W, Wang L, Murphy TL, Murphy KM, et al. BATF-JUN is critical for IRF4-mediated transcription in T cells. *Nature* (2012) 490:543–6. doi:10.1038/nature11530
142. Kwon H, Thierry-Mieg D, Thierry-Mieg J, Kim HP, Oh J, Tunyaplin C, et al. Analysis of interleukin-21-induced Prdm1 gene regulation reveals functional

- cooperation of STAT3 and IRF4 transcription factors. *Immunity* (2009) 31:941–52. doi:10.1016/j.immuni.2009.10.008
143. Gupta S, Jiang M, Anthony A, Pernis AB. Lineage-specific modulation of interleukin 4 signaling by interferon regulatory factor 4. *J Exp Med* (1999) 190:1837–48. doi:10.1084/jem.190.12.1837
 144. Krishnamoorthy V, Kannanganat S, Maienschein-Cline M, Cook SL, Chen J, Bahroos N, et al. The IRF4 gene regulatory module functions as a read-write integrator to dynamically coordinate T helper cell fate. *Immunity* (2017) 47:481–97.e7. doi:10.1016/j.immuni.2017.09.001
 145. Nakayama S, Poholek AC, Lu KT, Takahashi H, Kato M, Iwata S, et al. Type I IFN induces binding of STAT1 to Bcl6: divergent roles of STAT family transcription factors in the T follicular helper cell genetic program. *J Immunol* (2014) 192:2156–66. doi:10.4049/jimmunol.1300675
 146. Zhong Z, Wen Z, Darnell JE Jr. Stat3: a STAT family member activated by tyrosine phosphorylation in response to epidermal growth factor and interleukin-6. *Science* (1994) 264:95–8. doi:10.1126/science.8140422
 147. Asao H, Okuyama C, Kumaki S, Ishii N, Tsuchiya S, Foster D, et al. Cutting edge: the common gamma-chain is an indispensable subunit of the IL-21 receptor complex. *J Immunol* (2001) 167:1–5. doi:10.4049/jimmunol.167.1.1
 148. Read KA, Powell MD, Baker CE, Sreekumar BK, Ringel-Scaia VM, Bachus H, et al. Integrated STAT3 and IKaros zinc finger transcription factor activities regulate Bcl-6 expression in CD4(+) Th cells. *J Immunol* (2017) 199:2377–87. doi:10.4049/jimmunol.1700106
 149. Ma CS, Avery DT, Chan A, Batten M, Bustamante J, Boisson-Dupuis S, et al. Functional STAT3 deficiency compromises the generation of human T follicular helper cells. *Blood* (2012) 119:3997–4008. doi:10.1182/blood-2011-11-392985
 150. Schmitt N, Morita R, Bourdery L, Benteib SE, Zurawski SM, Banchereau J, et al. Human dendritic cells induce the differentiation of interleukin-21-producing T follicular helper-like cells through interleukin-12. *Immunity* (2009) 31:158–69. doi:10.1016/j.immuni.2009.04.016
 151. Weinstein JS, Laidlaw BJ, Lu Y, Wang JK, Schulz VP, Li N, et al. STAT4 and T-bet control follicular helper T cell development in viral infections. *J Exp Med* (2018) 215:337–55. doi:10.1084/jem.2017045702062018c
 152. Auderset F, Coutaz M, Tacchini-Cottier F. The role of Notch in the differentiation of CD4(+) T helper cells. *Curr Top Microbiol Immunol* (2012) 360:115–34. doi:10.1007/82_2012_227
 153. Auderset F, Schuster S, Fasnacht N, Coutaz M, Charmoy M, Koch U, et al. Notch signaling regulates follicular helper T cell differentiation. *J Immunol* (2013) 191:2344–50. doi:10.4049/jimmunol.1300643
 154. Dell'Aringa M, Reinhardt RL. Notch signaling represents an important checkpoint between follicular T-helper and canonical T-helper 2 cell fate. *Mucosal Immunol* (2018) 11:1079–91. doi:10.1038/s41385-018-0012-9
 155. Vaeth M, Muller G, Stauss D, Dietz L, Klein-Hessling S, Serfling E, et al. Follicular regulatory T cells control humoral autoimmunity via NFAT2-regulated CXCR5 expression. *J Exp Med* (2014) 211:545–61. doi:10.1084/jem.20130604
 156. Martinez GJ, Hu JK, Pereira RM, Crampton JS, Togher S, Bild N, et al. Cutting edge: NFAT transcription factors promote the generation of follicular helper T cells in response to acute viral infection. *J Immunol* (2016) 196:2015–9. doi:10.4049/jimmunol.1501841
 157. Liu X, Chen X, Zhong B, Wang A, Wang X, Chu F, et al. Transcription factor achaete-scute homologue 2 initiates follicular T-helper-cell development. *Nature* (2014) 507:513–8. doi:10.1038/nature12910
 158. Choi YS, Gullicksrud JA, Xing S, Zeng Z, Shan Q, Li F, et al. LEF-1 and TCF-1 orchestrate T(FH) differentiation by regulating differentiation circuits upstream of the transcriptional repressor Bcl6. *Nat Immunol* (2015) 16:980–90. doi:10.1038/ni.3226
 159. Wu T, Shin HM, Moseman EA, Ji Y, Huang B, Harly C, et al. TCF1 is required for the T follicular helper cell response to viral infection. *Cell Rep* (2015) 12:2099–110. doi:10.1016/j.celrep.2015.08.049
 160. Xu L, Cao Y, Xie Z, Huang Q, Bai Q, Yang X, et al. The transcription factor TCF-1 initiates the differentiation of T(FH) cells during acute viral infection. *Nat Immunol* (2015) 16:991–9. doi:10.1038/ni.3229
 161. Weber BN, Chi AW, Chavez A, Yashiro-Ohtani Y, Yang Q, Shestova O, et al. A critical role for TCF-1 in T-lineage specification and differentiation. *Nature* (2011) 476:63–8. doi:10.1038/nature10279
 162. Yu S, Zhou X, Steinke FC, Liu C, Chen SC, Zagorodna O, et al. The TCF-1 and LEF-1 transcription factors have cooperative and opposing roles in T cell development and malignancy. *Immunity* (2012) 37:813–26. doi:10.1016/j.immuni.2012.08.009
 163. Ogbe A, Miao T, Symonds AL, Omodho B, Singh R, Bhullar P, et al. Early growth response genes 2 and 3 regulate the expression of Bcl6 and differentiation of T follicular helper cells. *J Biol Chem* (2015) 290:20455–65. doi:10.1074/jbc.M114.634816
 164. Stauss D, Brunner C, Berberich-Siebelt F, Hopken UE, Lipp M, Muller G. The transcriptional coactivator Bob1 promotes the development of follicular T helper cells via Bcl6. *EMBO J* (2016) 35:881–98. doi:10.15252/embj.201591459
 165. Barnett LG, Simkins HM, Barnett BE, Korn LL, Johnson AL, Wherry EJ, et al. B cell antigen presentation in the initiation of follicular helper T cell and germinal center differentiation. *J Immunol* (2014) 192:3607–17. doi:10.4049/jimmunol.1301284
 166. Schwickert TA, Victora GD, Fooksman DR, Kamphorst AO, Mugnier MR, Gitlin AD, et al. T cell-limited checkpoint regulates affinity-dependent B cell entry into the germinal center. *J Exp Med* (2011) 208:1243–52. doi:10.1084/jem.20102477
 167. Serre K, Mohr E, Benezech C, Bird R, Khan M, Caamano JH, et al. Selective effects of NF-kappaB1 deficiency in CD4(+) T cells on Th2 and TFH induction by alum-precipitated protein vaccines. *Eur J Immunol* (2011) 41:1573–82. doi:10.1002/eji.201041126
 168. Hu H, Wu X, Jin W, Chang M, Cheng X, Sun SC. Noncanonical NF-kappaB regulates inducible costimulator (ICOS) ligand expression and T follicular helper cell development. *Proc Natl Acad Sci U S A* (2011) 108:12827–32. doi:10.1073/pnas.1105774108
 169. Xiao N, Eto D, Elly C, Peng G, Crotty S, Liu YC. The E3 ubiquitin ligase Itch is required for the differentiation of follicular helper T cells. *Nat Immunol* (2014) 15:657–66. doi:10.1038/ni.2912
 170. Wang H, Geng J, Wen X, Bi E, Kossenkova AV, Wolf AI, et al. The transcription factor Foxp1 is a critical negative regulator of the differentiation of follicular helper T cells. *Nat Immunol* (2014) 15:667–75. doi:10.1038/ni.2890
 171. Lee JY, Skon CN, Lee YJ, Oh S, Taylor JJ, Malhotra D, et al. The transcription factor KLF2 restrains CD4(+) T follicular helper cell differentiation. *Immunity* (2015) 42:252–64. doi:10.1016/j.immuni.2015.01.013
 172. Johnston RJ, Choi YS, Diamond JA, Yang JA, Crotty S. STAT5 is a potent negative regulator of TFH cell differentiation. *J Exp Med* (2012) 209:243–50. doi:10.1084/jem.20111174
 173. Park HJ, Kim DH, Choi JY, Kim WJ, Kim JY, Senejani AG, et al. PPARgamma negatively regulates T cell activation to prevent follicular helper T cells and germinal center formation. *PLoS One* (2014) 9:e99127. doi:10.1371/journal.pone.0099127
 174. Vanderleyden I, Linterman MA, Smith KG. Regulatory T cells and control of the germinal centre response. *Arthritis Res Ther* (2014) 16:471. doi:10.1186/s13075-014-0471-7
 175. Wollenberg I, Agua-Doce A, Hernandez A, Almeida C, Oliveira VG, Faro J, et al. Regulation of the germinal center reaction by Foxp3+ follicular regulatory T cells. *J Immunol* (2011) 187:4553–60. doi:10.4049/jimmunol.1101328
 176. Zhu Y, Zou L, Liu YC. T follicular helper cells, T follicular regulatory cells and autoimmunity. *Int Immunol* (2016) 28:173–9. doi:10.1093/intimm/dxv079
 177. Dhaeze T, Stinissen P, Liston A, Hellings N. Humoral autoimmunity: a failure of regulatory T cells? *Autoimmun Rev* (2015) 14:735–41. doi:10.1016/j.autrev.2015.04.006
 178. Sage PT, Sharpe AH. T follicular regulatory cells in the regulation of B cell responses. *Trends Immunol* (2015) 36:410–8. doi:10.1016/j.it.2015.05.005
 179. Xu L, Huang Q, Wang H, Hao Y, Bai Q, Hu J, et al. The kinase mTORC1 promotes the generation and suppressive function of follicular regulatory T cells. *Immunity* (2017) 47:538–551.e5. doi:10.1016/j.immuni.2017.08.011
 180. Chang JH, Hu H, Jin J, Puebla-Osorio N, Xiao Y, Gilbert BE, et al. TRAF3 regulates the effector function of regulatory T cells and humoral immune responses. *J Exp Med* (2014) 211:137–51. doi:10.1084/jem.20131019
 181. Jandl C, Liu SM, Canete PF, Warren J, Hughes WE, Vogelzang A, et al. IL-21 restricts T follicular regulatory T cell proliferation through Bcl-6 mediated inhibition of responsiveness to IL-2. *Nat Commun* (2017) 8:14647. doi:10.1038/ncomms14647

182. Maceiras AR, Fonseca VR, Agua-Doce A, Graca L. T follicular regulatory cells in mice and men. *Immunology* (2017) 152:25–35. doi:10.1111/imm.12774
183. Sage PT, Alvarez D, Godec J, von Andrian UH, Sharpe AH. Circulating T follicular regulatory and helper cells have memory-like properties. *J Clin Invest* (2014) 124:5191–204. doi:10.1172/JCI76861
184. Vignali DA, Collison LW, Workman CJ. How regulatory T cells work. *Nat Rev Immunol* (2008) 8:523–32. doi:10.1038/nri2343
185. Laidlaw BJ, Lu Y, Amezcua RA, Weinstein JS, Vander Heiden JA, Gupta NT, et al. Interleukin-10 from CD4(+) follicular regulatory T cells promotes the germinal center response. *Sci Immunol* (2017) 2:eaa4767. doi:10.1126/sciimmunol.aan4767
186. Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. *Science* (2003) 299:1057–61. doi:10.1126/science.1079490
187. Mayer CT, Floess S, Baru AM, Lahl K, Huehn J, Sparwasser T. CD8+ Foxp3+ T cells share developmental and phenotypic features with classical CD4+ Foxp3+ regulatory T cells but lack potent suppressive activity. *Eur J Immunol* (2011) 41:716–25. doi:10.1002/eji.201040913
188. Joosten SA, van Meijgaarden KE, Savage ND, de Boer T, Triebel F, van der Wal A, et al. Identification of a human CD8+ regulatory T cell subset that mediates suppression through the chemokine CC chemokine ligand 4. *Proc Natl Acad Sci U S A* (2007) 104:8029–34. doi:10.1073/pnas.0702257104
189. Kim HJ, Verbrinnen B, Tang X, Lu L, Cantor H. Inhibition of follicular T-helper cells by CD8(+) regulatory T cells is essential for self tolerance. *Nature* (2010) 467:328–32. doi:10.1038/nature09370
190. Kim HJ, Wang X, Radfar S, Sproule TJ, Roopenian DC, Cantor H. CD8+ T regulatory cells express the Ly49 class I MHC receptor and are defective in autoimmune prone B6-Yaa mice. *Proc Natl Acad Sci U S A* (2011) 108:2010–5. doi:10.1073/pnas.1018974108
191. Taghavi-Moghadam PL, Waseem TC, Hattler J, Glenn LM, Dobrian AD, Kaplan MH, et al. STAT4 regulates the CD8(+) regulatory T cell/T follicular helper cell axis and promotes atherogenesis in insulin-resistant Ldlr(-/-) mice. *J Immunol* (2017) 199:3453–65. doi:10.4049/jimmunol.1601429
192. Endharti AT, Rifa IM, Shi Z, Fukuoaka Y, Nakahara Y, Kawamoto Y, et al. Cutting edge: CD8+CD122+ regulatory T cells produce IL-10 to suppress IFN-gamma production and proliferation of CD8+ T cells. *J Immunol* (2005) 175:7093–7. doi:10.4049/jimmunol.175.11.7093
193. Holderried TA, Kim H-J, Lang PA, Cantor H. CD8+ Treg – from mouse to man. *Blood* (2013) 122:3474.
194. Miles B, Miller SM, Folkvord JM, Levy DN, Rakasz EG, Skinner PJ, et al. Follicular regulatory CD8 T cells impair the germinal center response in SIV and ex vivo HIV infection. *PLoS Pathog* (2016) 12:e1005924. doi:10.1371/journal.ppat.1005924
195. Rampuria P, Lang ML. CD1d-dependent expansion of NKT follicular helper cells in vivo and in vitro is a product of cellular proliferation and differentiation. *Int Immunol* (2015) 27:253–63. doi:10.1093/intimm/dxv007
196. Chang PP, Barral P, Fitch J, Pratama A, Ma CS, Kallies A, et al. Identification of Bcl-6-dependent follicular helper NKT cells that provide cognate help for B cell responses. *Nat Immunol* (2011) 13:35–43. doi:10.1038/ni.2166
197. Rampuria P, Lang GA, Devera TS, Gilmore C, Ballard JD, Lang ML. Coordination between T helper cells, iNKT cells, and their follicular helper subsets in the humoral immune response against Clostridium difficile toxin B. *J Leukoc Biol* (2017) 101:567–76. doi:10.1189/jlb.4A0616-271R
198. Lang ML. The influence of invariant natural killer T cells on humoral immunity to T-dependent and -independent antigens. *Front Immunol* (2018) 9:305. doi:10.3389/fimmu.2018.00305
199. Tonti E, Fedeli M, Napolitano A, Iannaccone M, von Andrian UH, Guidotti LG, et al. Follicular helper NKT cells induce limited B cell responses and germinal center formation in the absence of CD4(+) T cell help. *J Immunol* (2012) 188:3217–22. doi:10.4049/jimmunol.1103501
200. Fukazawa Y, Lum R, Okoye AA, Park H, Matsuda K, Bae JY, et al. B cell follicle sanctuary permits persistent productive simian immunodeficiency virus infection in elite controllers. *Nat Med* (2015) 21:132–9. doi:10.1038/nm.3781
201. Perdomo-Celis F, Taborda NA, Rugeles MT. Follicular CD8(+) T cells: origin, function and importance during HIV infection. *Front Immunol* (2017) 8:1241. doi:10.3389/fimmu.2017.01241
202. Quigley ME, Gonzalez VD, Granath A, Andersson J, Sandberg JK. CXCR5+ CCR7- CD8 T cells are early effector memory cells that infiltrate tonsil B cell follicles. *Eur J Immunol* (2007) 37:3352–62. doi:10.1002/eji.200636746
203. He R, Hou S, Liu C, Zhang A, Bai Q, Han M, et al. Follicular CXCR5-expressing CD8(+) T cells curtail chronic viral infection. *Nature* (2016) 537:412–28. doi:10.1038/nature19317
204. Im SJ, Hashimoto M, Gerner MY, Lee J, Kissick HT, Burger MC, et al. Defining CD8+ T cells that provide the proliferative burst after PD-1 therapy. *Nature* (2016) 537:417–21. doi:10.1038/nature19330
205. Leong YA, Chen Y, Ong HS, Wu D, Man K, Deleage C, et al. CXCR5(+) follicular cytotoxic T cells control viral infection in B cell follicles. *Nat Immunol* (2016) 17:1187–96. doi:10.1038/ni.3543
206. Li S, Folkvord JM, Rakasz EG, Abdelaal HM, Wagstaff RK, Kovacs KJ, et al. Simian immunodeficiency virus-producing cells in follicles are partially suppressed by CD8+ cells in vivo. *J Virol* (2016) 90:11168–80. doi:10.1128/JVI.01332-16
207. Petrovas C, Ferrando-Martinez S, Gerner MY, Casazza JP, Pegu A, Deleage C, et al. Follicular CD8 T cells accumulate in HIV infection and can kill infected cells in vitro via bispecific antibodies. *Sci Transl Med* (2017) 9:eag2285. doi:10.1126/scitranslmed.aag2285
208. Ferrando-Martinez S, Moysi E, Pegu A, Andrews S, Nganou Makamdop K, Ambrozak D, et al. Accumulation of follicular CD8+ T cells in pathogenic SIV infection. *J Clin Invest* (2018) 128:2089–103. doi:10.1172/JCI96207
209. Rahman MA, McKinnon KM, Karpova TS, Ball DA, Venzon DJ, Fan W, et al. Associations of simian immunodeficiency virus (SIV)-specific follicular CD8+ T cells with other follicular T cells suggest complex contributions to SIV viremia control. *J Immunol* (2018) 200:2714. doi:10.4049/jimmunol.1701403
210. Curran MA, Montalvo W, Yagita H, Allison JP. PD-1 and CTLA-4 combination blockade expands infiltrating T cells and reduces regulatory T and myeloid cells within B16 melanoma tumors. *Proc Natl Acad Sci U S A* (2010) 107:4275–80. doi:10.1073/pnas.0915174107
211. Jin Y, Lang C, Tang J, Geng J, Song HK, Sun Z, et al. CXCR5(+)CD8(+) T cells could induce the death of tumor cells in HBV-related hepatocellular carcinoma. *Int Immunopharmacol* (2017) 53:42–8. doi:10.1016/j.intimp.2017.10.009
212. Xu H, Liu J, Cui X, Zuo Y, Zhang Z, Li Y, et al. Increased frequency of circulating follicular helper T cells in lupus patients is associated with autoantibody production in a CD40L-dependent manner. *Cell Immunol* (2015) 295:46–51. doi:10.1016/j.cellimm.2015.01.014
213. Kawamoto M, Harigai M, Hara M, Kawaguchi Y, Tezuka K, Tanaka M, et al. Expression and function of inducible co-stimulator in patients with systemic lupus erythematosus: possible involvement in excessive interferon-gamma and anti-double-stranded DNA antibody production. *Arthritis Res Ther* (2006) 8:R62. doi:10.1186/ar1928
214. Wang L, Zhao P, Ma L, Shan Y, Jiang Z, Wang J, et al. Increased interleukin 21 and follicular helper T-like cells and reduced interleukin 10+ B cells in patients with new-onset systemic lupus erythematosus. *J Rheumatol* (2014) 41:1781–92. doi:10.3899/jrheum.131025
215. Terrier B, Costedoat-Chalumeau N, Garrido M, Geri G, Rosenzweig M, Musset L, et al. Interleukin 21 correlates with T cell and B cell subset alterations in systemic lupus erythematosus. *J Rheumatol* (2012) 39:1819–28. doi:10.3899/jrheum.120468
216. Lan Y, Luo B, Wang JL, Jiang YW, Wei YS. The association of interleukin-21 polymorphisms with interleukin-21 serum levels and risk of systemic lupus erythematosus. *Gene* (2014) 538:94–8. doi:10.1016/j.gene.2014.01.012
217. Wong CK, Wong PT, Tam LS, Li EK, Chen DP, Lam CW. Elevated production of B cell chemokine CXCL13 is correlated with systemic lupus erythematosus disease activity. *J Clin Immunol* (2010) 30:45–52. doi:10.1007/s10875-009-9325-5
218. Lee J, Shin EK, Lee SY, Her YM, Park MK, Kwok SK, et al. Oestrogen up-regulates interleukin-21 production by CD4(+) T lymphocytes in patients

- with systemic lupus erythematosus. *Immunology* (2014) 142:573–80. doi:10.1111/imm.12263
219. Nakou M, Papadimitrakaki ED, Fanouriakis A, Bertsias GK, Choulaki C, Goulidaki N, et al. Interleukin-21 is increased in active systemic lupus erythematosus patients and contributes to the generation of plasma B cells. *Clin Exp Rheumatol* (2013) 31:172–9.
 220. Liarski VM, Kaverina N, Chang A, Brandt D, Yanez D, Talasnik L, et al. Cell distance mapping identifies functional T follicular helper cells in inflamed human renal tissue. *Sci Transl Med* (2014) 6:230ra46. doi:10.1126/scitranslmed.3008146
 221. Chang A, Henderson SG, Brandt D, Liu N, Guttikonda R, Hsieh C, et al. In situ B cell-mediated immune responses and tubulointerstitial inflammation in human lupus nephritis. *J Immunol* (2011) 186:1849–60. doi:10.4049/jimmunol.1001983
 222. Vinuesa CG, Cook MC, Angelucci C, Athanasopoulos V, Rui L, Hill KM, et al. A RING-type ubiquitin ligase family member required to repress follicular helper T cells and autoimmunity. *Nature* (2005) 435:452–8. doi:10.1038/nature03555
 223. Linterman MA, Rigby RJ, Wong RK, Yu D, Brink R, Cannons JL, et al. Follicular helper T cells are required for systemic autoimmunity. *J Exp Med* (2009) 206:561–76. doi:10.1084/jem.20081886
 224. Bubier JA, Sproule TJ, Foreman O, Spolski R, Shaffer DJ, Morse HC III, et al. A critical role for IL-21 receptor signaling in the pathogenesis of systemic lupus erythematosus in BXSB-Yaa mice. *Proc Natl Acad Sci U S A* (2009) 106:1518–23. doi:10.1073/pnas.0807309106
 225. Mandik-Nayak L, Seo SJ, Sokol C, Potts KM, Bui A, Erikson J. MRL-lpr/lpr mice exhibit a defect in maintaining developmental arrest and follicular exclusion of anti-double-stranded DNA B cells. *J Exp Med* (1999) 189:1799–814. doi:10.1084/jem.189.11.1799
 226. Odegard JM, Marks BR, DiPlacido LD, Poholek AC, Kono DH, Dong C, et al. ICOS-dependent extrafollicular helper T cells elicit IgG production via IL-21 in systemic autoimmunity. *J Exp Med* (2008) 205:2873–86. doi:10.1084/jem.20080840
 227. Simpson N, Gatenby PA, Wilson A, Malik S, Fulcher DA, Tangye SG, et al. Expansion of circulating T cells resembling follicular helper T cells is a fixed phenotype that identifies a subset of severe systemic lupus erythematosus. *Arthritis Rheum* (2010) 62:234–44. doi:10.1002/art.25032
 228. Bohnhorst JO, Bjorgaard MB, Thoen JE, Jonsson R, Natvig JB, Thompson KM. Abnormal B cell differentiation in primary Sjogren's syndrome results in a depressed percentage of circulating memory B cells and elevated levels of soluble CD27 that correlate with serum IgG concentration. *Clin Immunol* (2002) 103:79–88. doi:10.1006/clim.2002.5199
 229. Szabo K, Papp G, Barath S, Gyimesi E, Szanto A, Zeher M. Follicular helper T cells may play an important role in the severity of primary Sjögren's syndrome. *Clin Immunol* (2013) 147:95–104. doi:10.1016/j.clim.2013.02.024
 230. Bombardieri M, Barone F, Humby F, Kelly S, McGurk M, Morgan P, et al. Activation-induced cytidine deaminase expression in follicular dendritic cell networks and interfollicular large B cells supports functionality of ectopic lymphoid neogenesis in autoimmune sialoadenitis and MALT lymphoma in Sjogren's syndrome. *J Immunol* (2007) 179:4929–38. doi:10.4049/jimmunol.179.7.4929
 231. Sidiropoulos PI, Boumpas DT. Lessons learned from anti-CD40L treatment in systemic lupus erythematosus patients. *Lupus* (2004) 13:391–7. doi:10.1191/0961203304lu1032oa
 232. Pertovaara M, Silvennoinen O, Isomaki P. Cytokine-induced STAT1 activation is increased in patients with primary Sjogren's syndrome. *Clin Immunol* (2016) 165:60–7. doi:10.1016/j.clim.2016.03.010
 233. Linsley PS, Nadler SG. The clinical utility of inhibiting CD28-mediated costimulation. *Immunol Rev* (2009) 229:307–21. doi:10.1111/j.1600-065X.2009.00780.x
 234. Patakas A, Ji RR, Weir W, Connolly SE, Benson RA, Nadler SG, et al. Abatacept inhibition of T cell priming in mice by induction of a unique transcriptional profile that reduces their ability to activate antigen-presenting cells. *Arthritis Rheumatol* (2016) 68:627–38. doi:10.1002/art.39470
 235. Verstappen GM, Meiners PM, Corneth OBJ, Visser A, Arends S, Abdulahad WH, et al. Attenuation of follicular helper T cell-dependent B cell hyperactivity by abatacept treatment in primary Sjogren's syndrome. *Arthritis Rheumatol* (2017) 69:1850–61. doi:10.1002/art.40165
 236. Wang L, Zhang Y, He M. Clinical predictors for the prognosis of myasthenia gravis. *BMC Neurol* (2017) 17:77. doi:10.1186/s12883-017-0857-7
 237. Luo C, Li Y, Liu W, Feng H, Wang H, Huang X, et al. Expansion of circulating counterparts of follicular helper T cells in patients with myasthenia gravis. *J Neuroimmunol* (2013) 256:55–61. doi:10.1016/j.jneuroim.2012.12.001
 238. Le Coz C, Joublin A, Pasquali JL, Korganow AS, Dumortier H, Monneaux F. Circulating TFH subset distribution is strongly affected in lupus patients with an active disease. *PLoS One* (2013) 8:e75319. doi:10.1371/journal.pone.0075319
 239. Romme Christensen J, Bornsen L, Ratzner R, Piehl F, Khademi M, Olsson T, et al. Systemic inflammation in progressive multiple sclerosis involves follicular T-helper, Th17- and activated B-cells and correlates with progression. *PLoS One* (2013) 8:e57820. doi:10.1371/journal.pone.0057820
 240. Liu R, Wu Q, Su D, Che N, Chen H, Geng L, et al. A regulatory effect of IL-21 on T follicular helper-like cell and B cell in rheumatoid arthritis. *Arthritis Res Ther* (2012) 14:R255. doi:10.1186/ar4100
 241. An LM, Li J, Ji LL, Li GT, Zhang ZL. [Detection of peripheral follicular helper T cells in rheumatoid arthritis]. *Beijing Da Xue Xue Bao Yi Xue Ban* (2016) 48:951–7. doi:10.3969/j.issn.1671-167X.2016.06.006
 242. Rao DA, Gurish MF, Marshall JL, Slowikowski K, Fonseka CY, Liu Y, et al. Pathologically expanded peripheral T helper cell subset drives B cells in rheumatoid arthritis. *Nature* (2017) 542:110–4. doi:10.1038/nature20810
 243. Moschovakis GL, Bubke A, Friedrichsen M, Falk CS, Feederle R, Förster R. T cell specific Cxcr5 deficiency prevents rheumatoid arthritis. *Sci Rep* (2017) 7:8933. doi:10.1038/s41598-017-08935-6
 244. Kenefick R, Wang CJ, Kapadi T, Wardzinski L, Attridge K, Clough LE, et al. Follicular helper T cell signature in type 1 diabetes. *J Clin Invest* (2015) 125:292–303. doi:10.1172/JCI76238
 245. Xu X, Shi Y, Cai Y, Zhang Q, Yang F, Chen H, et al. Inhibition of increased circulating Tfh cell by anti-CD20 monoclonal antibody in patients with type 1 diabetes. *PLoS One* (2013) 8:e79858. doi:10.1371/journal.pone.0079858
 246. Ferreira RC, Simons HZ, Thompson WS, Cutler AJ, Dopico XC, Smyth DJ, et al. IL-21 production by CD4(+) effector T cells and frequency of circulating follicular helper T cells are increased in type 1 diabetes patients. *Diabetologia* (2015) 58:781–90. doi:10.1007/s00125-015-3509-8
 247. Viisanen T, Ihantola E-L, Nääntö-Salonen K, Hyöty H, Nurminen N, Selvenius J, et al. Circulating CXCR5+PD-1+ICOS+ follicular T helper cells are increased close to the diagnosis of type 1 diabetes in children with multiple autoantibodies. *Diabetes* (2017) 66:437–47. doi:10.2337/db16-0714
 248. Arif S, Leete P, Nguyen V, Marks K, Nor NM, Estorninho M, et al. Blood and islet phenotypes indicate immunological heterogeneity in type 1 diabetes. *Diabetes* (2014) 63:3835–45. doi:10.2337/db14-0365
 249. Zhou J, Wang Y, He Y, Gao Y, Wan R, Cai M, et al. Non-obese type 2 diabetes patients present intestinal B cell dysregulations associated with hyperactive intestinal Tfh cells. *Mol Immunol* (2018) 97:27–32. doi:10.1016/j.molimm.2018.03.008
 250. Gaddis DE, Padgett LE, Wu R, McSkimming C, Romines V, Taylor AM, et al. Apolipoprotein AI prevents regulatory to follicular helper T cell switching during atherosclerosis. *Nat Commun* (2018) 9:1095. doi:10.1038/s41467-018-03493-5
 251. Clement M, Guedj K, Andreatta F, Morvan M, Bey L, Khallou-Laschet J, et al. Control of the T follicular helper-germinal center B-cell axis by CD8(+) regulatory T cells limits atherosclerosis and tertiary lymphoid organ development. *Circulation* (2015) 131:560–70. doi:10.1161/CIRCULATIONAHA.114.010988
 252. Kamekura R, Shigehara K, Miyajima S, Jitsukawa S, Kawata K, Yamashita K, et al. Alteration of circulating type 2 follicular helper T cells and regulatory B cells underlies the comorbid association of allergic rhinitis with bronchial asthma. *Clin Immunol* (2015) 158:204–11. doi:10.1016/j.clim.2015.02.016
 253. Jin J, Kobayashi T, Bachman KA, Kita H. T follicular helper (Tfh) cells are activated by natural exposure to pollens during the Ragweed Hay fever season. *J Allergy Clin Immunol* (2018) 141:AB176. doi:10.1016/j.jaci.2017.12.561

254. Dolence JJ, Kobayashi T, Iijima K, Krempski J, Drake LY, Dent AL, et al. Airway exposure initiates peanut allergy by involving the IL-1 pathway and T follicular helper cells in mice. *J Allergy Clin Immunol* (2017). doi:10.1016/j.jaci.2017.11.020
255. Akdis CA, Akdis M, Bieber T, Bindslev-Jensen C, Boguniewicz M, Eigenmann P, et al. Diagnosis and treatment of atopic dermatitis in children and adults: European Academy of Allergology and Clinical Immunology/American Academy of Allergy, Asthma and Immunology/PRACTALL Consensus Report. *J Allergy Clin Immunol* (2006) 118:152–69. doi:10.1016/j.jaci.2006.03.045
256. Eyerich K, Novak N. Immunology of atopic eczema: overcoming the Th1/Th2 paradigm. *Allergy* (2013) 68:974–82. doi:10.1111/all.12184
257. Werfel T, Allam JB, Biedermann T, Eyerich K, Gilles S, Guttman-Yassky E, et al. Cellular and molecular immunologic mechanisms in patients with atopic dermatitis. *J Allergy Clin Immunol* (2016) 138:336–49. doi:10.1016/j.jaci.2016.06.010
258. Eyerich K, Huss-Marp J, Darsow U, Wollenberg A, Foerster S, Ring J, et al. Pollen grains induce a rapid and biphasic eczematous immune response in atopic eczema patients. *Int Arch Allergy Immunol* (2008) 145:213–23. doi:10.1159/000109290
259. Szabó K, Gáspár K, Dajnoki Z, Papp G, Fábos B, Szegedi A, et al. Expansion of circulating follicular T helper cells associates with disease severity in childhood atopic dermatitis. *Immunol Lett* (2017) 189:101–8. doi:10.1016/j.imlet.2017.04.010
260. Czarnecki T, Esaki H, Gonzalez J, Renert-Yuval Y, Brunner P, Oliva M, et al. Alterations in B-cell subsets in pediatric patients with early atopic dermatitis. *J Allergy Clin Immunol* (2017) 140:134–44.e9. doi:10.1016/j.jaci.2016.09.060
261. Ballesteros-Tato A, Randall TD, Lund FE, Spolski R, Leonard WJ, León B. T follicular helper cell plasticity shapes pathogenic T helper 2 cell-mediated immunity to inhaled house dust mite. *Immunity* (2016) 44:259–73. doi:10.1016/j.immuni.2015.11.017
262. Ma CS, Nichols KE, Tangye SG. Regulation of cellular and humoral immune responses by the SLAM and SAP families of molecules. *Annu Rev Immunol* (2007) 25:337–79. doi:10.1146/annurev.immunol.25.022106.141651
263. Ma CS, Hare NJ, Nichols KE, Dupré L, Andolfi G, Roncarolo M-G, et al. Tangye. Impaired humoral immunity in X-linked lymphoproliferative disease is associated with defective IL-10 production by CD4(+) T cells. *J Clin Invest* (2005) 115:1049–59. doi:10.1172/JCI200523139
264. Schmitt N, Bustamante J, Bourdery L, Bentebibel SE, Boisson-Dupuis S, Hamlin F, et al. IL-12 receptor beta1 deficiency alters in vivo T follicular helper cell response in humans. *Blood* (2013) 121:3375–85. doi:10.1182/blood-2012-08-448902
265. Lindqvist M, van Lunzen J, Soghoian DZ, Kuhl BD, Ranasinghe S, Kranias G, et al. Expansion of HIV-specific T follicular helper cells in chronic HIV infection. *J Clin Invest* (2012) 122:3271–80. doi:10.1172/JCI64314
266. Petrovas C, Yamamoto T, Gerner MY, Boswell KL, Wloka K, Smith EC, et al. CD4 T follicular helper cell dynamics during SIV infection. *J Clin Invest* (2012) 122:3281–94. doi:10.1172/JCI63039
267. Perreau M, Savoye AL, De Crignis E, Corpataux JM, Cubas R, Haddad EK, et al. Follicular helper T cells serve as the major CD4 T cell compartment for HIV-1 infection, replication, and production. *J Exp Med* (2013) 210:143–56. doi:10.1084/jem.20121932
268. Xu Y, Weatherall C, Bailey M, Alcantara S, De Rose R, Estaquier J, et al. Simian immunodeficiency virus infects follicular helper CD4 T cells in lymphoid tissues during pathogenic infection of pigtail macaques. *J Virol* (2013) 87:3760–73. doi:10.1128/JVI.02497-12
269. Xu Y, Phetsouphanh C, Suzuki K, Aggrawal A, Graff-Dubois S, Roche M, et al. HIV-1 and SIV predominantly use CCR5 expressed on a precursor population to establish infection in T follicular helper cells. *Front Immunol* (2017) 8:376. doi:10.3389/fimmu.2017.00376
270. Hufert FT, van Lunzen J, Janossy G, Bertram S, Schmitz J, Haller O, et al. Germinal centre CD4+ T cells are an important site of HIV replication in vivo. *AIDS* (1997) 11:849–57. doi:10.1097/00002030-199707000-00003
271. Kohler SL, Pham MN, Folkvord JM, Arends T, Miller SM, Miles B, et al. Germinal center T follicular helper cells are highly permissive to HIV-1 and alter their phenotype during virus replication. *J Immunol* (2016) 196:2711–22. doi:10.4049/jimmunol.1502174
272. Connick E, Mattila T, Folkvord JM, Schlichtemeier R, Meditz AL, Ray MG, et al. CTL fail to accumulate at sites of HIV-1 replication in lymphoid tissue. *J Immunol* (2007) 178:6975–83. doi:10.4049/jimmunol.178.11.6975
273. Hong JJ, Amancha PK, Rogers K, Ansari AA, Villinger F. Spatial alterations between CD4(+) T follicular helper, B, and CD8(+) T cells during simian immunodeficiency virus infection: T/B cell homeostasis, activation, and potential mechanism for viral escape. *J Immunol* (2012) 188:3247–56. doi:10.4049/jimmunol.1103138
274. Lu J, Lv Y, Lv Z, Xu Y, Huang Y, Cui M, et al. Expansion of circulating T follicular helper cells is associated with disease progression in HIV-infected individuals. *J Infect Public Health* (2018). doi:10.1016/j.jiph.2018.01.005
275. De Milito A, Nilsson A, Titanji K, Thorstensson R, Reizenstein E, Narita M, et al. Mechanisms of hypergammaglobulinemia and impaired antigen-specific humoral immunity in HIV-1 infection. *Blood* (2004) 103:2180–6. doi:10.1182/blood-2003-07-2375
276. Hart M, Steel A, Clark SA, Moyle G, Nelson M, Henderson DC, et al. Loss of discrete memory B cell subsets is associated with impaired immunization responses in HIV-1 infection and may be a risk factor for invasive pneumococcal disease. *J Immunol* (2007) 178:8212–20. doi:10.4049/jimmunol.178.12.8212
277. Cubas RA, Mudd JC, Savoye A-L, Perreau M, van Grevenynghe J, Metcalf T, et al. Inadequate T follicular cell help impairs B cell immunity during HIV infection. *Nat Med* (2013) 19:494–9. doi:10.1038/nm.3109
278. Moir S, Fauci AS. B cells in HIV infection and disease. *Nat Rev Immunol* (2009) 9:235–45. doi:10.1038/nri2524
279. Fahey LM, Wilson EB, Elsaesser H, Fistonich CD, McGavern DB, Brooks DG. Viral persistence redirects CD4 T cell differentiation toward T follicular helper cells. *J Exp Med* (2011) 208:987–99. doi:10.1084/jem.20101773
280. Harker JA, Lewis GM, Mack L, Zuniga EI. Late interleukin-6 escalates T follicular helper cell responses and controls a chronic viral infection. *Science* (2011) 334:825–9. doi:10.1126/science.1208421
281. Herati RS, Muselman A, Vella L, Bengsch B, Parkhouse K, Del Alcazar D, et al. Successive annual influenza vaccination induces a recurrent oligoclonotypic memory response in circulating T follicular helper cells. *Sci Immunol* (2017) 2:eag2152. doi:10.1126/sciimmunol.aag2152
282. Koutsakos M, Wheatley AK, Loh L, Clemens EB, Sant S, Nussing S, et al. Circulating TFH cells, serological memory, and tissue compartmentalization shape human influenza-specific B cell immunity. *Sci Transl Med* (2018) 10:eaan8405. doi:10.1126/scitranslmed.aan8405
283. Wang R, Xie R, Song Z. Circulating regulatory Tfh cells are enriched in patients with chronic hepatitis B infection and induce the differentiation of regulatory B cells. *Exp Cell Res* (2018) 365:171–6. doi:10.1016/j.yexcr.2018.02.031
284. Rodrigues V, Laforge M, Campillo-Gimenez L, Soundaramourty C, Correia-de-Oliveira A, Dinis-Oliveira RJ, et al. Abortive T follicular helper development is associated with a defective humoral response in *Leishmania infantum*-infected macaques. *PLoS Pathog* (2014) 10:e1004096. doi:10.1371/journal.ppat.1004096
285. Haben I, Hartmann W, Breloer M. Nematode-induced interference with vaccination efficacy targets follicular T helper cell induction and is preserved after termination of infection. *PLoS Negl Trop Dis* (2014) 8:e3170. doi:10.1371/journal.pntd.0003170
286. Perez-Mazliah D, Nguyen MP, Hosking C, McLaughlin S, Lewis MD, Tumwine I, et al. Follicular helper T cells are essential for the elimination of *Plasmodium* infection. *EBioMedicine* (2017) 24:216–30. doi:10.1016/j.ebiom.2017.08.030
287. de Leval L, Rickman DS, Thielen C, Reynies A, Huang YL, Delsol G, et al. The gene expression profile of nodal peripheral T-cell lymphoma demonstrates a molecular link between angioimmunoblastic T-cell lymphoma (AITL) and follicular helper T (TFH) cells. *Blood* (2007) 109:4952–63. doi:10.1182/blood-2006-10-055145
288. Grogg KL, Attygalle AD, Macon WR, Remstein ED, Kurtin PJ, Dogan A. Expression of CXCL13, a chemokine highly upregulated in germinal center T-helper cells, distinguishes angioimmunoblastic T-cell lymphoma from

- peripheral T-cell lymphoma, unspecified. *Mod Pathol* (2006) 19:1101–7. doi:10.1038/modpathol.3800625
289. Vinuesa CG, Tangye SG, Moser B, Mackay CR. Follicular B helper T cells in antibody responses and autoimmunity. *Nat Rev Immunol* (2005) 5:853–65. doi:10.1038/nri1714
 290. Cortes JR, Ambesi-Impiombato A, Couronné L, Quinn SA, Kim CS, da Silva Almeida AC, et al. RHOA G17V induces T follicular helper cell specification and promotes lymphomagenesis. *Cancer Cell* (2018) 33:259–73. e7. doi:10.1016/j.ccell.2018.01.001
 291. Ochando J, Braza MS. T follicular helper cells: a potential therapeutic target in follicular lymphoma. *Oncotarget* (2017) 8:112116–31. doi:10.18632/oncotarget.22788
 292. Gu-Trantien C, Loi S, Garaud S, Equeter C, Libin M, de Wind A, et al. CD4+ follicular helper T cell infiltration predicts breast cancer survival. *J Clin Invest* (2013) 123:2873–92. doi:10.1172/JCI67428
 293. Bindea G, Mlecnik B, Tosolini M, Kirilovsky A, Waldner M, Obenauf AC, et al. Spatiotemporal dynamics of intratumoral immune cells reveal the immune landscape in human cancer. *Immunity* (2013) 39:782–95. doi:10.1016/j.immuni.2013.10.003
 294. Wang Z, Wang Z, Diao Y, Qian X, Zhu N, Dong W. Circulating follicular helper T cells in Crohn's disease (CD) and CD-associated colorectal cancer. *Tumour Biol* (2014) 35:9355–9. doi:10.1007/s13277-014-2208-2
 295. Cha Z, Zang Y, Guo H, Rechlic JR, Olsanova LM, Gu H, et al. Association of peripheral CD4+ CXCR5+ T cells with chronic lymphocytic leukemia. *Tumour Biol* (2013) 34:3579–85. doi:10.1007/s13277-013-0937-2
 296. Shi W, Li X, Cha Z, Sun S, Wang L, Jiao S, et al. Dysregulation of circulating follicular helper T cells in nonsmall cell lung cancer. *DNA Cell Biol* (2014) 33:355–60. doi:10.1089/dna.2013.2332
 297. Xiao H, Luo G, Son H, Zhou Y, Zheng W. Upregulation of peripheral CD4+CXCR5+ T cells in osteosarcoma. *Tumour Biol* (2014) 35:5273–9. doi:10.1007/s13277-014-1686-6

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2018 Qin, Waseem, Sahoo, Bieerkehazhi, Zhou, Galkina and Nurieva. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



T Cells That Help B Cells in Chronically Inflamed Tissues

Deepak A. Rao*

Division of Rheumatology, Immunology, Allergy, Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, MA, United States

OPEN ACCESS

Edited by:

Maria Pia Cicalese,
San Raffaele Scientific Institute
(IRCCS), Italy

Reviewed by:

Constantinos Petrovas,
National Institutes of Health (NIH),
United States
Thomas Ciucci,
National Cancer Institute (NCI),
United States

*Correspondence:

Deepak A. Rao
darao@bwh.harvard.edu

Specialty section:

This article was submitted to
T Cell Biology,
a section of the journal
Frontiers in Immunology

Received: 30 May 2018

Accepted: 06 August 2018

Published: 23 August 2018

Citation:

Rao DA (2018) T Cells That Help B
Cells in Chronically Inflamed Tissues.
Front. Immunol. 9:1924.
doi: 10.3389/fimmu.2018.01924

Chronically inflamed tissues commonly accrue lymphocyte aggregates that facilitate local T cell-B cell interactions. These aggregates can range from small, loosely arranged lymphocyte clusters to large, organized ectopic lymphoid structures. In some cases, ectopic lymphoid structures develop germinal centers that house prototypical T follicular helper (Tfh) cells with high expression of Bcl6, CXCR5, PD-1, and ICOS. However, in many chronically inflamed tissues, the T cells that interact with B cells show substantial differences from Tfh cells in their surface phenotypes, migratory capacity, and transcriptional regulation. This review discusses observations from multiple diseases and models in which tissue-infiltrating T cells produce factors associated with B cell help, including IL-21 and the B cell chemoattractant CXCL13, yet vary dramatically in their resemblance to Tfh cells. Particular attention is given to the PD-1^{hi} CXCR5⁺ Bcl6^{low} T peripheral helper (Tph) cell population in rheumatoid arthritis, which infiltrates inflamed synovium through expression of chemokine receptors such as CCR2 and augments synovial B cell responses via CXCL13 and IL-21. The factors that regulate CD4⁺ T cell production of CXCL13 and IL-21 in these settings are also discussed. Understanding the range of T cell populations that can provide help to B cells within chronically inflamed tissues is essential to recognize these cells in diverse inflammatory conditions and to optimize either broad or selective therapeutic targeting of B cell-helper T cells.

Keywords: T follicular helper cells, T peripheral helper cells, IL-21, CXCL13, B cells, ectopic lymphoid structure, ectopic lymphoid follicle, tertiary lymphoid tissue

INTRODUCTION

CD4⁺ T cells play a critical role in stimulating effective B cell responses and production of high-affinity antibodies. T follicular helper (Tfh) cells are generally considered the dominant T cell population capable of providing help to B cells. The interactions between Tfh cells and B cells within follicles of secondary lymphoid organs (SLOs) occur with precise spatial and temporal coordination to yield productive antibody responses. Yet in both protective and pathologic immune responses, T cell-B cell interactions also occur outside of SLOs and within inflamed peripheral tissues. Interactions between T cells and B cells within peripheral tissues are much less well characterized, and the T cell populations that participate in these interactions can differ from Tfh cells in their surface features, migration patterns, and effector functions. This review examines the phenotypes of T cells within peripheral tissues that express factors associated with providing B cell help, in particular IL-21 and CXCL13. In many cases, the T cells that help B cells within peripheral tissues do not conform to the typical phenotype of active Tfh cells and often lack the signature features of Tfh cells such as high expression of CXCR5 and Bcl6. These observations underscore

that a broader definition of B cell-helper T cells is required to fully capture the range of T cells capable of productive T cell-B cell interactions, in particular during pathologic chronic immune responses.

THE T FOLLICULAR HELPER PARADIGM

The discovery and characterization of Tfh cells has helped define the paradigm of productive T cell-B cell interactions and has established Tfh cells as the prototype of a B cell-helper T cell (1). Tfh cells are drawn into lymphoid follicles through expression of CXCR5, a chemokine receptor shared with B cells that detects the chemokine CXCL13 (2–4). Expression of CXCR5 is a defining feature of Tfh cells, a logical association given that this chemokine receptor helps direct these cells to the location after which they are named (5–8). The transcription factor *Ascl2* enables early CXCR5 expression, which is then reinforced by the central Tfh transcription factor *Bcl6* (9–13). Upregulation of CXCR5 and downregulation of CCR7 induces migration of CXCR5⁺ T cells into follicles, where interactions with B cells enhance *Bcl6* expression and stabilize the Tfh cell phenotype, in part through ICOS-ICOSL interactions (10, 14–16).

Bcl6 is essential for the development and persistence of Tfh cells *in vivo* and promotes expression of many Tfh cell-associated factors, including CXCR5, ICOS, PD-1, and CXCL13, while suppressing alternative differentiation paths (10–13, 17). Human but not mouse Tfh cells produce large amounts of CXCL13, which helps to recruit CXCR5⁺ B cells to follicles (13, 18, 19). In addition, Tfh cells characteristically express IL-21, a cytokine that promotes B cell proliferation in germinal centers (GC) and differentiation into plasma cells (20–22). While there is heterogeneity in Tfh cell phenotypes and functions in SLOs, GC-Tfh display the most pronounced B cell-helper phenotype, with high expression of CXCR5, *Bcl6*, CXCL13, and IL-21 accompanied by high expression of the immunomodulatory receptors ICOS and PD-1 (1, 18, 20, 23). These 4 key features of GC-Tfh cells: (1) CXCR5 expression, (2) high *Bcl6* expression, (3) surface expression of PD-1 and ICOS, and (4) secretion of IL-21 and CXCL13, are commonly assayed in studies looking for Tfh-like cells at sites outside of SLOs, including blood and peripheral tissues.

T CELL-B CELL INTERACTIONS IN INFLAMED TISSUES

During an adaptive immune response, activated T cells differentiate into distinct effector populations that acquire specialized functions coupled with appropriate migratory programs. For example, activated effector or effector memory cells home to peripheral tissues to direct inflammatory responses, while CXCR5⁺ Tfh cells migrate to lymphoid follicles to help B cells (24). Migratory capacity sometimes serves as a defining feature of T cell populations: CCR7⁺ CD62L⁺ T central memory cells recirculate through SLOs, CCR7[−] CD62L[−] T effector memory cells traffic through peripheral tissues, and CD103⁺ CD69⁺ T resident memory cells localize to tissue

barriers (25). However, in pathologic conditions involving chronic inflammation, such as autoimmune diseases, cancer, and organ transplantation, the anatomic distinction between inflamed peripheral tissues and lymph node follicles begins to blur. Chronically inflamed sites frequently develop aggregates of T cells and B cells that promote B cell responses locally within the tissue (26). Often these aggregates appear as small, disorganized lymphocyte clusters. In some cases, the aggregates mature into organized ectopic lymphoid structures (ELS, also referred to as tertiary lymphoid organs/tissues/structures) that acquire many features of follicles in SLOs, including compartmentalization of T cell-rich and B cell-rich zones and accumulation of follicular dendritic cells (FDC) (26).

T cell-B cell interactions within chronically inflamed tissues can reproduce many of the key features of productive interactions within SLO follicles, including somatic hypermutation, class switching, and differentiation of plasma cells (26). For example, the inflamed synovium in rheumatoid arthritis (RA) develops lymphoid aggregates, which can range from small clusters to organized follicles with GCs (27). Plasma cells differentiate within these aggregates and are often seen extending out from the borders of the aggregates (28, 29). Similarly, somatic hypermutation and differentiation of plasmablasts occurs within tubulointerstitial aggregates in kidneys affected by lupus nephritis (30). Infiltrated tumors and rejecting kidney allografts also show evidence of lymphoid aggregates that support B cell somatic hypermutation despite the absence of typical GC (31–34). The accumulation of lymphocytes and plasma cells in chronically inflamed tissues occurs frequently enough to have merited its own term “lymphoplasmacytic infiltrate,” which appears not uncommonly in clinical histopathologic reports.

Defining the T cell populations most relevant for driving B cell aggregation and proliferation within peripheral tissues remains challenging. It has been generally assumed that Tfh cells infiltrate peripheral tissues to drive B cell responses within these tissues. However, this assumption requires some caution. For one, the migratory receptors required to infiltrate a peripheral tissue differ substantially from those required to access SLOs. CXCR5⁺ Tfh cells typically do not express chemokine receptors that recruit T cells to inflamed peripheral tissues, such as CCR2, CCR5, and CX3CR1 (1). Rather, a tightly controlled migratory program helps restrict Tfh cells to CXCL13-laden follicles. Thus it is not obvious how Tfh cells would be initially recruited to inflamed sites that lack well-established follicles. This raises the possibility that T cells with a distinct migratory capacity—directed by expression of a distinct cohort of migratory receptors—may interact with B cells in diffusely inflamed tissues.

Second, lymphocyte populations within inflamed tissues are dominated by memory cells that have undergone prior activation, with a smaller representation of naïve lymphocytes than in SLOs. Both memory T cells and B cells differ in their responsiveness to antigen-receptor activation, requirement for costimulation, and sensitivity to cytokines and other inputs as compared to their naïve counterparts (25, 35, 36); thus, the interactions between effector/memory T cells and B cells within inflamed tissues may differ from those that occur within follicles of SLOs. Even within the same tonsil, different B cell populations (naïve,

memory, GC-B cells) yield markedly different responses when co-cultured with distinct tonsil CD4⁺ T cell populations: GC-Tfh strongly promote immunoglobulin production from GC-B cells, yet ICOS^{low} CXCR5^{low} T cells fail to do so because the FasL that they produce kills co-cultured GC-B cells (23). In contrast, naïve and memory B cell populations respond well to CXCR5^{low} ICOS^{low} T cells because these B cell populations do not express Fas and are insensitive to FasL (23). Thus the rules of engagement may differ depending on the specific cell types present and the organization of the interactions.

Finally, determining the phenotype of T cells that help B cells in inflamed tissues faces technical challenges. The tissues that can be obtained are often small, with many fewer lymphocytes available compared to SLOs. In addition, the highly overlapping expression programs between Tfh cells and B cells limit interpretation of total tissue sample analyses (e.g., mRNA from the whole tissue) because one cannot distinguish whether key factors such as CXCR5 and Bcl6 are derived from the T cells or B cells. The tightly entangled interaction between T cells and B cells in tissues provides challenges for standard immunofluorescence microscopy and even laser capture microscopy (37). Immunohistochemistry and immunofluorescence microscopy allow visualization of T cell-B cell aggregates and may provide the resolution to discriminate T cell vs. B cell phenotypes. However, relatively few parameters are measured simultaneously, and discriminating cell surface protein expression (e.g., CXCR5 expression) on the surface of T cells surrounded by a tight cluster of CXCR5-bright B cells can be difficult. In addition, the cellular source of soluble factors such as IL-21 and CXCL13 can be ambiguous, especially for factors that bind to extracellular matrix (38–40). Utilization of single cell resolution analyses are therefore particularly valuable to interrogate in detail the phenotypes of potential T cells that help B cells within tissue samples.

B CELL-HELPER T CELLS IN RHEUMATOID ARTHRITIS

RA synovium provides a valuable case study to interrogate T cell-B cell interactions in a chronically inflamed tissue. RA is an autoimmune disease that prominently features pathologic T cell-B cell interactions. Approximately 2/3 of RA patients develop autoantibodies—antibodies against citrullinated proteins and/or other immunoglobulins (rheumatoid factors). Seropositive RA patients commonly develop T cell-B cell aggregates within synovial tissue (41). These are often small or medium-sized aggregates, although ~10–15% of patients develop more organized aggregates with features of GC (27, 42, 43). Most B cells in RA synovium are CD27⁺ memory B cells with mutated B cell receptor sequences, although a minority of naïve B cells can also be found within lymphoid aggregates (28, 44, 45). Synovial B cells produce RA-associated autoantibodies, and B cell receptor repertoire analyses suggest that memory B cells activated in synovial aggregates can differentiate into plasma cells locally within the tissue, even in the absence of GCs (28, 29, 46). Plasma cells are often found extending out from around lymphoid aggregates (29). In some patients, plasma cells

accumulate densely throughout the synovium, which is a defining feature of a highly inflamed RA synovium and can distinguish RA from other causes of early arthritis (47, 48).

Given the prominent lymphocyte aggregates and evidence of ongoing B cell activation, RA synovium appears a very likely place for accumulation of Tfh cells to help drive these responses. RA is somewhat unique among human autoimmune diseases in that relatively large samples of the target tissue can be obtained for research. Synovial tissue obtained either at the time of joint replacement surgery or through a research-protocol biopsy can provide sufficient material for multiple high-dimensional cellular analyses (41, 49). Surprisingly, a mass cytometry screen of T cells isolated from RA synovial tissue performed by our group revealed few CXCR5⁺ Tfh cells in RA synovium, despite frequent B cells and plasma cells (50). In addition, flow cytometry demonstrated an almost total absence of Tfh cells in RA synovial fluid. However, seropositive RA synovial tissue and fluid samples contained a large population of PD-1^{hi} CXCR5⁺ T cells, comprising ~25% of the CD4⁺ T cells, that expressed high levels of IL-21 and CXCL13 and induced memory B cell differentiation into plasma cells *in vitro*. CXCR5 expression was absent from PD-1^{hi} CXCR5⁺ cells at both the protein and mRNA level, in contrast to the well-detected CXCR5 expression in tonsil Tfh cells and circulating Tfh cells. PD-1^{hi} CXCR5⁺ T cells were observed adjacent to B cells both within lymphocyte aggregates and more diffusely throughout inflamed synovium. Like Tfh cells, synovial PD-1^{hi} CXCR5⁺ T cells also expressed high levels of ICOS and MAF, a transcription factor that promotes IL-21 production (discussed below). Cytometric and transcriptomic comparisons of PD-1^{hi} CXCR5⁺ and PD-1^{hi} CXCR5⁺ cells from blood showed high expression of TIGIT, SAP, CD200, and SLAM and low expression of CD25 and CD127 in both populations (50).

However, PD-1^{hi} CXCR5⁺ T cells also showed key differences from Tfh cells. Unlike Tfh cells, PD-1^{hi} CXCR5⁺ cells from synovium did not express high levels of Bcl6, and instead they showed elevated levels of the counter-regulator Blimp1, which opposes the actions of Bcl6 (10). In addition, PD-1^{hi} cells from synovium not only lacked CXCR5, but they frequently expressed a different cohort of chemokine receptors, including CCR2, CCR5, and CX3CR1, with approximately half of the PD-1^{hi} cells expressing CCR2 (50). Notably, these receptors are abundant on leukocytes that infiltrate RA synovium, which contains high levels of the CCR2 ligand CCL2 (MCP-1) and the CCR5 ligand RANTES, among others (51–55). These chemokines are so prominent in inflamed synovium that attempts have been made to interfere with both CCR2- and CCR5-mediated trafficking therapeutically to blunt synovium inflammation, although such approaches have not yet succeeded perhaps due to migratory signal redundancy or incomplete signal blockade (56–58).

PERIPHERAL VS. FOLLICULAR B CELL-HELPER T CELLS

The presence of a B cell-helper T cell population in RA synovium with a CXCR5⁺ Bcl6^{low} but CCR2⁺ Blimp1⁺ phenotype illustrates that IL-21, CXCL13, and B cell help can be provided by

T cell populations that differ substantially in phenotype from Tfh cells (**Figure 1**). Two key points arise from these observations:

- 1) *T cells that help B cells do not necessarily express CXCR5*. The RA synovium observations reveal a population of T cells that home to sites of inflammation, using chemokine receptors that target these sites, and help B cells within these sites. We have suggested that these CXCR5[−] PD-1^{hi} T cells can be considered peripheral, rather than follicular, B cell-helper T cells, and we referred to these cells as T peripheral helper (Tph) cells for shorthand (50). Once activated within inflamed tissues, Tph cells may secrete CXCL13 to recruit B cells (and potentially Tfh cells) and produce IL-21 to help drive B cell survival and maturation.
- 2) *T cells that help B cells do not necessarily express high levels of Bcl6*. The Tph cells in RA synovium express modest levels of Bcl6, well below that seen in synovial CXCR5⁺ PD-1^{hi} Tfh cells, indicating that B cell-helper function can be dissociated from high Bcl6 expression. As discussed below, Bcl6 does not directly control production of IL-21 (11, 13). Rather, one major role for high Bcl6 levels is directing the positioning of Tfh cells in follicles through upregulation of CXCR5 and downregulation of CCR7, PSGL1, and EBI2 (13, 17). High Bcl6 expression also helps CXCR5⁺ cytotoxic CD8⁺ T cells localize to follicles (59). This role of Bcl6 in directing T cells to follicles may be less important for B cell-helper T cells that are present in more diffusely infiltrated tissues without true GC. Bcl6 also importantly controls additional features of Tfh cells, including expression of molecules involved in interacting with B cells (ICOS, PD-1, CD40L); however, it is possible that the lower levels of Bcl6, such as seen in synovial Tph cells, may be

sufficient for this function. Other transcription factors such as MAF may also co-regulate some of these molecules (13).

B cell-helper T cells have been reported within target tissues in range of autoimmune, inflammatory, and malignant conditions; it will be of substantial interest to now clarify the extent to which these cells resemble Tfh cells vs. Tph cells in each condition. There is no doubt that Tfh cells play a critical role in providing help to B cells in SLOs, and it is clear Tfh cells infiltrate well-formed ELS with GC in inflamed tissues as well. Yet the following sections highlight selected diseases and models in which evidence suggests the presence of potential B cell-helper T cells—identified by production of IL-21 or CXCL13—that differ from prototypical Tfh cells. For this discussion, Tfh cells are defined as CXCR5⁺ Bcl6⁺ CD4⁺ T cell that produce IL-21 (and CXCL13 in humans), while cells that lack CXCR5 or Bcl6 are considered non-Tfh cells and may represent candidate Tph cells.

IL-21-PRODUCING T CELLS IN INFLAMED TISSUES

Tfh cells produce high levels of IL-21 to stimulate B cell survival and differentiation, thus production of IL-21 is often used as a surrogate marker for Tfh cells. It is important to note that IL-21 can also be produced by other CD4⁺ T cell populations and NKT cells, and that this cytokine acts on many different target cell populations to enhance or tailor responses, for example boosting cytotoxicity of CD8⁺ T cells and NK cells, or altering macrophage function (22). Nonetheless, it seems likely that T cells that produce IL-21 while in close proximity to B cells,

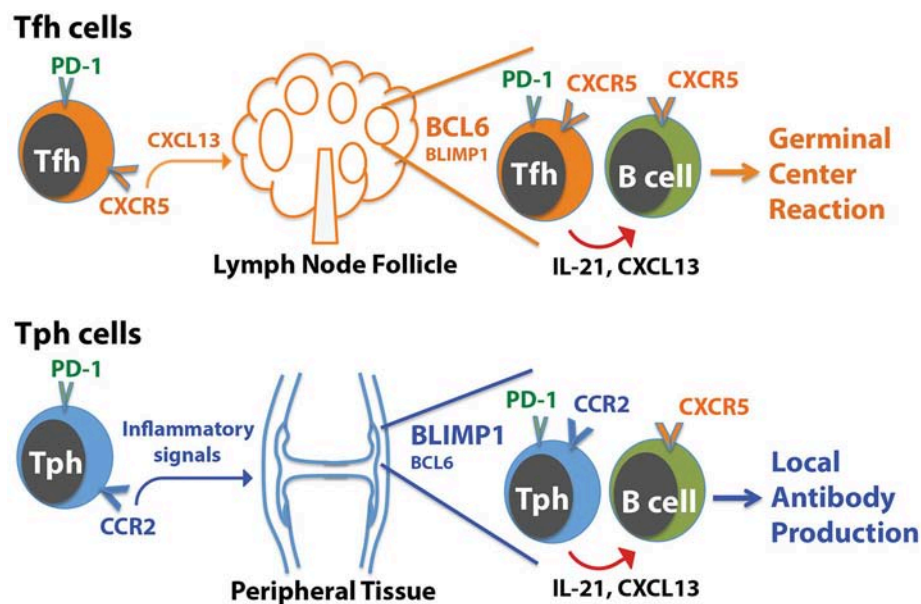


FIGURE 1 | Distinctions between Tph cells and Tfh cells. Tfh cells home to follicles following the chemokine CXCL13, which acts on CXCR5 on the Tfh cells. In contrast, Tph cells express a distinct set of chemokine receptors that direct migration to sites of inflammation, such as the RA joint. In both cases, Tph cells or Tfh cells interact with CXCR5⁺ B cells by producing CXCL13 to help recruit B cells, and IL-21 to help drive B cell activation and differentiation. For Tfh cells, this promotes generation of a GC reaction in follicles. For Tph cells, this may augment local antibody production within the tissue.

for example within lymphocyte aggregates, can enhance B cell responses. The following examples suggest that IL-21-producing CD4⁺ T cells in inflamed tissues can show substantial variability in their expression of Tfh-associated factors.

Transplanted renal and cardiac allografts undergoing rejection can develop ELS with plasma cells that produce donor-specific antibodies (60, 61). Renal allografts that undergo rapid rejection contain high mRNA expression of IL-21 (62). IL-21 expression levels correlate strongly with expression of AID, a protein required for somatic hypermutation and class switch recombination in B cells, suggesting a potential connection between T cell-derived IL-21 and B cell activation with the grafts (62). However, IL-21 expression levels in rejected allografts showed no correlation with Bcl6 expression levels, and fewer than 5% of the CD4⁺ T cells in rejected allografts expressed CXCR5⁺ by flow cytometry (62). An independent series of analyses of kidney allografts undergoing acute T cell-mediated rejection found that the majority of CD4⁺ T cells within lymphocyte aggregates expressed IL-21 by immunofluorescence microscopy, yet few of these T cells co-expressed Bcl6 (63, 64). These observations suggest that CD4⁺ T cells within lymphocyte aggregates of rejecting allografts produce abundant IL-21, yet the majority of this IL-21 appears to come from non-CXCR5⁺/Bcl6⁺ CD4⁺ T cells.

In multiple sclerosis, an autoimmune disease that involves infiltration of T cells and B cells in parenchymal demyelinating lesions, as well as development of lymphoid aggregates in the meninges, a similarly high frequency of IL-21-producing T cells has been reported (65–67). Immunofluorescence microscopy analyses suggested that more than half of CD4⁺ T cells in both active and chronic parenchymal lesions produce IL-21, while the receptor for IL-21 is expressed broadly on infiltrating B cells and T cells and on neurons (67). The frequency of CXCR5⁺ T cells in these lesions is not yet clear; however, in cerebrospinal fluid from multiple sclerosis patients ~20% of memory CD4⁺ T cells express CXCR5, comparable to the frequency observed in blood. While the expression of CXCR5 may differ between cerebrospinal fluid and tissue, these observations raise the possibility that a large portion of the IL-21⁺ CD4⁺ T cells in multiple sclerosis may not express CXCR5.

Analyses of nasal polyps that develop due to chronic upper airway inflammation indicated that ~20–40% of CD4⁺ T cells produce IL-21, as observed by both flow cytometry and immunohistochemistry (68–70). CXCR5⁺ effector memory T cells comprise the majority of the IL-21-producing cells in affected nasal tissues (69). These cells frequently express PD-1 and co-produce IFN- γ (69, 70). CXCR5⁺ Bcl6⁺ Tfh cells are enriched in the lymphoid aggregates that form in these polyps compared to healthy tissue; however, Bcl6 expression is relatively infrequent in nasal polyps overall (perhaps 2–5% of CD4⁺ T cells), with higher frequencies in T cells from polyps that contained GCs or eosinophils (68–70). Thus while it is clear that Tfh cells are enriched in nasal polyps, it is not yet clear that Tfh cells provide the majority of the B cell help in nasal polyps.

In contrast to RA and the conditions above, IgG4-related disease (IgG4-RD) provides an example in which *bona fide*

GC-Tfh cells appear to dominate the T cell infiltrate. IgG4-RD involves formation of large, fibrotic lesions that are heavily infiltrated by IgG4⁺ plasma cells (71). The lesions can form in several tissues including the salivary glands (sialadenitis) and lacrimal glands (dacryoadenitis) and contain large ELS with GC (72). The majority of T cells purified from these lesions show a prototypical GC-Tfh phenotype, with expression of CXCR5, Bcl6, PD-1, ICOS, CXCL13, and IL-21 at high levels that equal or exceed that seen in human tonsils (72, 73). Interestingly, GCs appear more common in IgG4-RD than in Sjogren's syndrome, an autoimmune disease that also produces lymphoid aggregates in the salivary glands but involves at least in part a CCR9⁺ B cell-helper T cell population with features distinct from Tfh cells (discussed below) (72, 74).

Murine models also support the idea that non-Tfh cells can provide important help to B cells via IL-21. In lupus-prone MRL/lpr mice, a population of CXCR4⁺ PSGL-1^{low} CD4⁺ T cells accumulates within extrafollicular foci in the spleen to support plasmablast responses (75). These extrafollicular helpers require Bcl6 and ICOS yet express little CXCR5 and do not transmigrate to CXCL13; rather, they express CXCR4, which may help position them in areas of higher CXCL12 concentration such as near the red pulp (75–77). These extrafollicular helper T cells provide a unique source of IL-21 to promote plasma cell generation and IgG production from B cells in extracellular follicles (75–77). A potentially comparable T cell population has also been observed in human tonsil, identified as CXCR5^{low} ICOS^{low} CD4⁺ T cells, although it is difficult to fully distinguish whether these cells are extrafollicular helpers or 'pre-Tfh' cells (23). *In vitro*, this CXCR5^{low} ICOS^{low} Bcl6^{low} population efficiently helps naïve and memory B cells via production of IL-21, yet appears to kill, rather than help, GC-B cells via Fas-FasL interactions (23).

Productive interactions between B cells and non-Tfh cells also occur in inflamed peripheral tissues in murine models. In a chronic lung inflammation model induced by ovalbumin + LPS, large lymphoid aggregates develop in the lungs (78). While occasional aggregates show features of ELS, most appear loose and poorly organized. Interestingly, B cells with GC features, including staining with peanut agglutinin and the monoclonal antibody GL7, were found both in the ELS and in the loose aggregates, suggesting development of GC-like B cells within the lung aggregates; however, no Bcl6⁺ or CXCR5⁺ T cells were present in these tissues (78). Rather, high IL-21 production and B cell-helper activity existed within the CXCR5⁺ Bcl6⁺ T cell population resident in the lung. While no defined markers were identified to specifically isolate the B cell-helper T cells out of the total lung T cell pool, high average expression of PD-1 and ICOS suggest a likely overlap with the Tph cell described in RA synovium. A separate study of a house dust mite-induced murine asthma model also identified a PD-1^{hi} CXCR5⁺ IL-21-producing CD4⁺ T cell population that accumulates in the lungs (79). This T cell population derived from Tfh cells in lymph nodes and migrated to the lungs to enhance eosinophilic airway inflammation via IL-21.

Observations from the non-obese diabetic (NOD) mouse provide further evidence of B cell help from non-Tfh cells. NOD mice develop lymphoid aggregates in the pancreas, with

development of more organized ELS with FDC networks over time (80). The pancreas in NOD mice is infiltrated with an IL-21-producing effector memory CD4⁺ T cell population with high ICOS⁺ expression. Strikingly, the majority of these cells expressed CCR9, a chemokine receptor that confers sensitivity to the chemokine CCL25 and helps mediate migration to mucosal sites such as the small bowel (81, 82). Pancreatic CCR9⁺ T cells expressed high levels of Bcl6 and MAF, yet little CXCR5 and SAP and variable PD-1. Consistent with IL-21 production, pancreatic CCR9⁺ T cells could enhance immunoglobulin production from co-cultured B cells *in vitro* (82). IL-21⁺ CCR9⁺ cells were also detected in the salivary glands of NOD mice (82). Consistent with these observations, CCR9⁺ ICOS⁺ cells are present in salivary glands of patients with Sjogren's syndrome, and the levels of the CCR9 ligand CCL25 in salivary tissue correlated positively with the levels of IL-21 and plasma cells in the tissue (83). In addition, human CCR9⁺ T cells from blood produced IL-21 at even higher levels than do CXCR5⁺ T cells and enhanced responses of co-cultured B cells *in vitro* (81, 83).

An independent study of NOD mice identified a potentially distinct population of B cell-helper T cells that is highly enriched in infiltrated salivary glands. This T cell population, identified as PD-1^{hi} ICOS^{hi} CD73^{hi} CD200^{hi}, produced high levels of IL-21 and IFN- γ , suggesting possible B cell-helper function, yet lacked Bcl6 and CXCR5 and also showed low expression of CCR9 (84). Viewed together, these observations suggest the tissues of Sjogren's syndrome patients and NOD mice accumulate IL-21-producing B cell-helper T cell populations that can lack typical Tfh-associated features such as Bcl6 and CXCR5.

Direct interrogation of Bcl6-deficient T cells provides further support for effective B cell help from non-Tfh cells. In a murine influenza model, Th1 CD4⁺ T cells can promote generation of neutralizing antibodies when Tfh cells are rendered absent due to CD3-specific deletion of Bcl6 (85). In this model, CXCR5⁻ Th1 cells secreted enough IL-21, co-produced with IFN- γ , to stimulate production of protective, albeit low-affinity, neutralizing antibodies to influenza virus. Bcl6-independent production of IL-21 by CD4⁺ T cells has also been observed in a murine *Plasmodium chabaudi* infection model, in which CD4-specific deletion of Bcl6 eliminated development of CXCR5^{hi} GC-Tfh cells but did not alter production of IL-21 in the spleen, which derived primarily from IL-21/IFN- γ double producers that retained intermediate CXCR5 expression (86).

REGULATION OF IL-21 PRODUCTION IN T CELLS

The above examples demonstrate that populations of T cells with varying degrees of similarity to Tfh cells can produce IL-21 and provide B cell help in inflamed tissues. The factors that regulate the ability of CD4⁺ T cells to produce IL-21 differ somewhat between human T cells and mouse T cells. In mice, IL-6 and IL-21 potently induce IL-21 production from T cells stimulated *in vitro*, and IL-21 is frequently detected in cultured

of T cells stimulated under Th17-polarizing conditions (22, 87–89). In contrast, IL-6 and IL-21 do not efficiently induce IL-21 production by human CD4⁺ T cells; IL-12 is far more potent in promoting IL-21 production by human T cells and often induces IFN- γ /IL-21 co-producers (90, 91). IL-12 can also enhance IL-21 production by murine CD4⁺ T cells, which transit through an early Tfh-like phenotype when stimulated under Th1-inducing conditions (92). In addition, IL-12 transiently enhances expression of Bcl6, CXCR5, and ICOS via STAT4; this effect is subsequently suppressed by Tbet (92). The overlap between Tfh and Th1 features in T cells early after activation appears to fit well with the theme that IL-21-producing T cells in inflamed tissues often also show Th1 features and co-produce IFN- γ .

Importantly, transcriptional control of IL-21 production occurs largely independently of Bcl6. Murine CD4⁺ T cells that lack Bcl6 produce IL-21 at normal levels, and overexpression of Bcl6 in murine T cells does not increase IL-21 production (11). Similarly, overexpression of Bcl6 in human tonsil CD4⁺ T cells does not alter IL-21 production (13). Instead, the transcription factor MAF, which is highly expressed in both Tfh cells and synovial Tph cells, appears most directly associated with IL-21 production. *In vitro*, MAF can activate an IL-21 promoter-luciferase reporter construct, unlike Tbet or GATA3, and overexpression of MAF in human tonsil CD4⁺ T cells or mouse naïve CD4⁺ T cells increases IL-21 production (13, 93, 94). In addition, genetic deletion of MAF reduces IL-21 production from murine Th17 cells (95). Interestingly, in human T cells, the combination of IL-12 plus TGF- β induces several features of Tfh cells, including increased expression of IL-21, MAF, Bcl6, and CXCR5 (91). This occurs as TGF- β directs the actions of STAT4 and STAT3, activated by IL-12, to enhance Tfh cell features (91). TGF- β also induces MAF in murine CD4⁺ T cells, yet it concurrently inhibits IL-21 production and does not promote a Tfh phenotype in murine T cells (91, 93). A more comprehensive delineation of the signals that control IL-21 production, along with other factors required for cell-surface interactions with B cells, will be valuable to better understand the regulation of T cell-B cell interactions in the periphery.

CXCL13-PRODUCING T CELLS IN INFLAMED TISSUES

While IL-21 can be produced broadly by different T cell populations and may act on diverse target cells, production of CXCL13 appears to be a much more specific marker of T cell-B cell interactions. CXCL13 acts specifically to recruit and position CXCR5⁺ cells within lymphoid follicles, primarily B cells and Tfh cells (2–4, 8, 96). Overexpression of CXCL13 in a peripheral tissue induces formation of B cell aggregates that eventually mature into ELS, while neutralization of CXCL13 in murine models reduces lymphoid aggregate formation and tissue inflammation severity in several chronic inflammation models (97–103). Consistent with these observations, the highest expression of CXCR5 in RA synovium is found on B cells, with bright CXCR5 expression seen on B cells within synovial aggregates (50).

Importantly, Tfh cells from humans and primates, but not rodents, produce large amounts of CXCL13 (1, 7, 18). In lymph nodes, GC-Tfh cells are an important, and perhaps primary, source of CXCL13. Immunohistochemistry of tonsils shows co-staining of CXCL13 primarily with PD-1⁺ T cells, rather than CD21⁺ FDCs, within follicles (104, 105). Intracellular flow cytometry of human inguinal lymph node cells also showed expression of CXCL13 primarily in PD-1^{hi} CXCR5^{hi} GC-Tfh cells, with few CXCL13⁺ cells among other T cell populations, B cells, or stromal cells (106). Further, the frequency of GC-Tfh cells in lymph nodes correlates positively with serum CXCL13 levels in both humans and non-human primates, such that the serum CXCL13 level has been suggested to represent a biomarker of total GC activity. If cells produce CXCL13 in order to recruit B cells (which express high levels of CXCR5, the only known receptor for CXCL13), then CXCL13-producing T cells (GC-Tfh cells and others) are prime candidates to interact with B cells in various settings.

While GC-Tfh cells are a major source of CXCL13 in lymph nodes, there are accumulating data that non-Tfh cells with varied surface phenotypes can produce CXCL13 at different sites. In Sjogren's syndrome patients, CCR9⁺ CXCR5⁺ T cells from the blood produced CXCL13 (in addition to IL-21 as described above), albeit at lower levels than was produced by CXCR5⁺ cells (83). CXCL13 production from CD4⁺ T cells in RA synovium was first described by Manzo et al., who noted CXCL13⁺ CD4⁺ T cells both in areas of large lymphoid aggregates and also in areas with smaller aggregates or diffuse lymphocyte infiltration (107). Flow cytometry of synovial fluid T cells revealed that most CXCL13⁺ T cells are memory CD4⁺ T cells that lack CXCR5 and contain little Bcl6. These CXCL13⁺ T cells were often CD27 negative, suggesting a chronically activated or terminally differentiated phenotype (107). An independent study identified the same population of CXCL13⁺ CD4⁺ T cells with little CXCR5 or Bcl6 expression, but with high expression of PD-1 (108). Detailed assessments by mass cytometry, RNA-seq transcriptomics, and *in vitro* B cell-helper assays then defined this synovial T cell population as Tph cells (50).

Single cell RNA-seq analyses of synovial tissue have recently highlighted Tph cells as the predominant source of CXCL13 in rheumatoid synovium. The vast majority of CXCL13 signal in RA synovium detected by single cell RNA-seq is in the Tph cell population, with little CXCL13 detected in synovial fibroblasts, macrophages, vascular cells, or B cells (109). This powerful analysis provides a high-resolution view of the potential cellular sources of CXCL13 in the tissue, which can be difficult to discern from immunohistochemical analysis given that CXCL13 can deposit on matrix components once secreted (40). CXCL13 has emerged as a promising biomarker in RA, with its level in the synovium associated with the presence of synovial lymphoid aggregates, autoantibody positivity, and erosive disease (110, 111). In this context, it is remarkable that the majority of this CXCL13 appears to come from infiltrating Tph cells (109). Notably, CXCL13 production by human B cell-helper T cells stands in stark contrast to the murine Tfh cells, which do not make CXCL13. Rather, stromal and parenchymal cells appear to be the major producers of CXCL13 in murine tissues, stimulated

by lymphotoxin α/β and TNF in SLOs or IL-17A in inflamed tissues (112, 113).

Single cell RNA-seq analyses appear to be particularly sensitive in identifying CXCL13⁺ T cell populations, perhaps because CXCL13 mRNA transcripts are expressed at very high levels in restricted subpopulations of cells (50, 109). RNA-seq of single cell T cells sorted from hepatocellular carcinoma samples identified an easily distinguished CXCL13⁺ CD4⁺ T cell population isolated from tumor tissue (114). The chemokine receptor expression on this T cell population (e.g., CXCR5) was not evaluated in this study; however, an independent study of T cell infiltrates in hepatocellular carcinoma identified a prominent IL-21-producing T cell population that accumulates within the peritumoral stroma (115). These cells, which are CXCR5⁺ PD-1^{low}, comprise ~10% of tumor CD4⁺ T cells, and their frequency correlates positively with plasma cell infiltrates and negatively with overall survival (115). Whether the CXCL13⁺ cells seen by RNA-seq and IL-21⁺ T cells detected by flow cytometry represent the same T cell population in hepatocellular carcinoma will be of interest to resolve.

Detailed studies of breast cancer tissue have also revealed a population of infiltrating CXCL13⁺ CD4⁺ T cells (105, 116). In breast cancer tissue, CXCL13⁺ CD4⁺ T cells accumulate either within small B cell aggregates, adjacent to B cell follicles, or occasionally within B cell follicles (105, 116). These CXCL13⁺ T cells display a PD-1^{hi} ICOS^{mid} CXCR5⁺ surface phenotype and express high Tbet but relatively low Bcl6 levels compared to tonsil GC-Tfh cells (105). In addition to elevated CXCL13, these cells showed high expression of IL-21, IFN- γ , and IL-10. The presence of CXCL13⁺ T cells in breast cancer tissue was found to be a good prognostic sign, associated with a higher rate of disease-free survival (105). While most CXCL13⁺ T cells in breast cancer samples are CD4⁺ T cells, a subset of tumor-infiltrating CD8⁺ T cells was also noted to produce CXCL13 and reside near B cell follicles, albeit with lower CXCL13 expression than in CD4⁺ T cells (105). CXCL13 expression has also been observed in CD103⁺ CD8⁺ T cells in ovarian cancer tumors (117). It will be interest to evaluate further the relative contributions, and functional overlap, of CD4⁺ and CD8⁺ T cells that produce CXCL13. Notably, CD4⁺ T cells with B cell-helper features, including high expression of PD-1, ICOS, and CXCL13, are enriched in multiple solid tumors and in murine tumor model, and these cells are preferentially expanded by anti-CTLA4 but not anti-PD-1 therapy (118–120). Clarifying the beneficial vs. deleterious roles of these cells in anti-tumor immune responses will be important in order to utilize this population as a predictive biomarker.

REGULATION OF CXCL13 PRODUCTION IN T CELLS

Compared to other effector functions, relatively little is known about the regulation of CXCL13 production in human CD4⁺ T cells. Triggering of the T cell receptor plus the CD28 costimulatory receptor on synovial CD4⁺ T cells augments

CXCL13 secretion (107). Secretion of CXCL13 by synovial T cells appears relatively long-lived and can be sustained for several days by the addition of TNF + IL-6 to *in vitro* cultures (108). CXCL13-producing synovial CD4⁺ T cells show a stable phenotype and are able to retain the ability to produce CXCL13 for several weeks in culture (108). Interestingly, overexpression of Bcl6 enhances production of CXCL13 production by human tonsil CD4⁺ T cells, suggesting that Bcl6 controls not only the localization of Tfh cells, but also their ability to direct co-localization of B cells (13). Recruitment of B cells through CXCL13 production may act in synergy with upregulation of molecules involved in the T cell-B cell interaction, such as CD40L, PD-1, and ICOS, by Bcl6 (13).

Signals that induce naïve CD4⁺ T cells to acquire the ability to produce CXCL13 are not well defined. TGF- β appears important, as treatment of naïve CD4⁺T cells with TGF- β during *in vitro* stimulation promotes CXCL13 production (105, 121). Activin A, which signals through the Activin A receptor to activate Smad2-Smad3 pathways in a manner similar to TGF- β , also promotes CXCL13 production (122). CXCL13 production has also been detected from *in vitro* differentiated Th17 clones, which may be a consequence of the TGF- β used in the Th17 differentiation culture conditions (123). In addition, as with differentiation of mouse Tfh cells, IL-2-induced activation of STAT5 inhibits the

CXCL13-producing phenotype in human CD4⁺ T cells (105, 121).

TPH CELLS AS POTENTIAL INSTIGATORS OF ELS

Production of CXCL13 by T cells that infiltrate an inflamed peripheral tissue may be a key step in the initiation of ELS formation, providing an early stimulus to recruit CXCR5⁺ B cells (Figure 2). Proposed here is a model in which Tph cells infiltrate a site of peripheral inflammation, drawn in by chemokines such as a CCL2, CCL5, and CX3CL1. Upon encountering antigen plus inflammatory cytokines within the tissue, Tph cells produce large amounts of CXCL13, which help recruit CXCR5⁺ B cells into the tissue and draw them into close proximity with the Tph cells. This process may induce formation of the small lymphocyte aggregates frequently observed in inflamed tissues. Recognition of antigen presented on antigen presenting cells within the tissue, including perhaps on recruited B cells, also induces Tph cell production of IL-21, which helps drive maturation and survival of local B cells. If the reaction occurs vigorously enough, then continued production of CXCL13 and IL-21 will recruit and

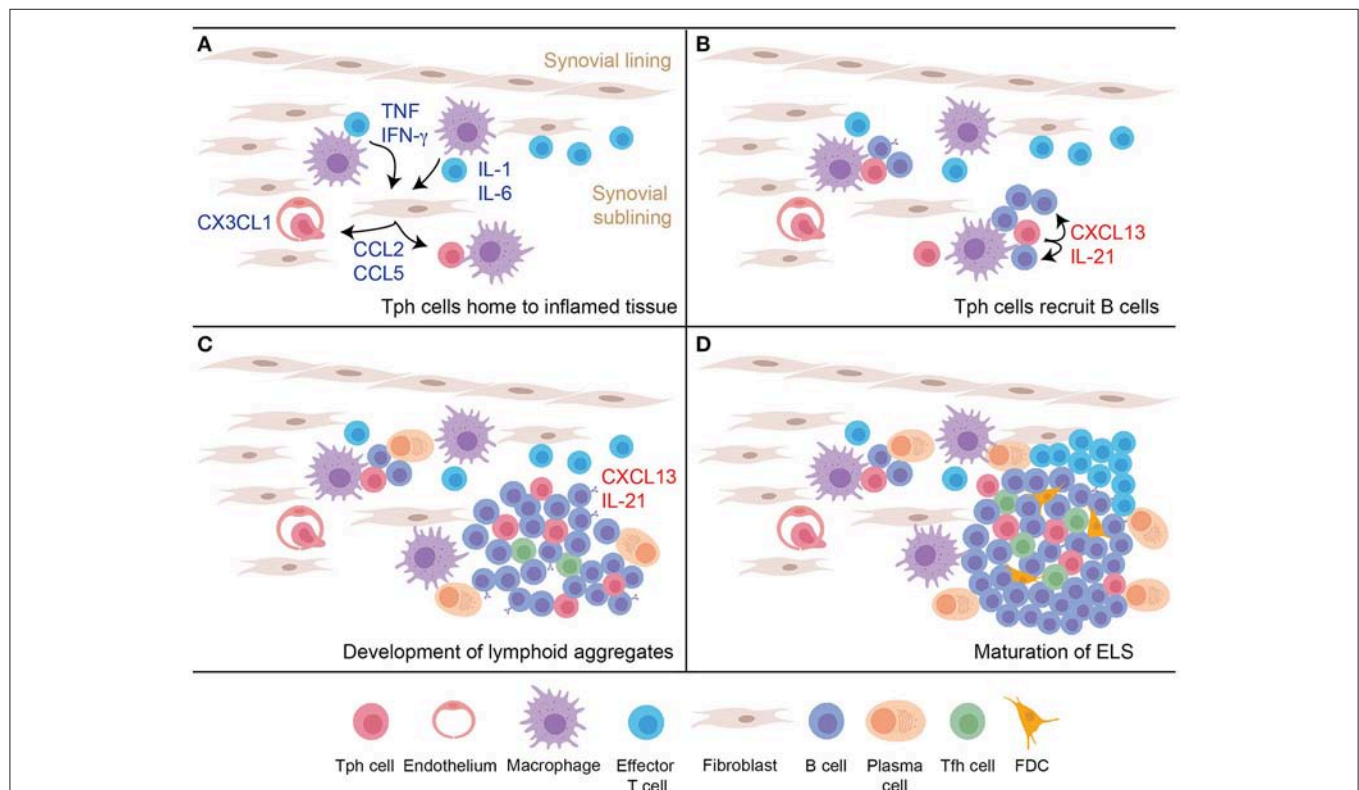


FIGURE 2 | A model for nucleation of ELS in inflamed tissues by Tph cells. **(A)** An inflammatory response in the peripheral tissue induces production of chemokines and additional signals to recruit peripheral-homing Tph cells. **(B)** After infiltrating the tissue, activated Tph cells produce CXCL13 to recruit B cells, and produce IL-21 to promote B cell activation and survival. **(C)** Continued activation of Tph cells and interaction with B cells leads to development of loose lymphoid aggregates, which support some plasma cell differentiation. Tfh cells begin to be recruited into these aggregates in part due to CXCL13 production. **(D)** Ongoing T cell-B cell interactions and CXCL13 production leads to further maturation of the lymphoid aggregate into organized follicles that acquires features of GC, including accumulation of FDC.

sustain additional B cells and Tfh cells, as well as eventually FDCs, to yield development of mature ELS. In this model, disrupting the function of Tph cells may abrogate formation of both small and large lymphocyte aggregates within inflamed tissues.

QUESTIONS RAISED

Many questions remain to understand the range of B cell-helper T cells that drive B cell responses in peripheral tissues. T cells with Tfh-like features have been identified in target tissues in several diseases and models beyond those discussed here, including lupus, autoimmune hepatitis, primary biliary sclerosis, systemic sclerosis, Hashimoto's thyroiditis and several others (37, 124–127). Detailed phenotypic analyses of these cells in different inflammatory conditions will be of tremendous interest in understanding the features of B cell-helper T cells across diseases. For conditions in which both Tfh cells and Tph cells accumulate in inflamed tissues, the more challenging question will be to determine the relative roles, and potentially distinct functions, of these two B cell-helper populations in the involved tissue.

The developmental relationship between Tfh cells and Tph cells is also a key topic to be clarified. It is possible that a subset of Tfh cells differentiate into Tph cells during the GC response. Alternatively, Tph cells may derive from peripheral effector cells that acquire B cell-helper function. Sorted human CXCR5+ Tfh cells and CCR2+ Tph cells remain relatively distinct after short-term *in vitro* stimulation (50); however, the developmental relationships between these cells may be most definitively addressed in murine models. In addition, it will be of interest to consider where Tph cells fall within the range of B cell-helper T cells observed in SLOs, which include GC-Tfh, pre-Tfh, and extrafollicular helpers. For example, do these populations comprise a spectrum of B cell-helper cells distinguished by migratory programs, with Tph cells on one far end and GC-Tfh at the other? Additional questions raised by these observations include: Do Tph cells have a role in

protective immune responses, or do they primarily mediate chronic, pathologic T cell-B cell interactions? Can Tph cells or peripheral T cell-B cell interactions be specifically targeted to blunt pathologic inflammatory responses?

CONCLUSION

While much emphasis has been placed on the central role of Tfh cells in providing B cell help, the phenotypes of B cell-helper T cells in inflamed tissues can differ substantially from Tfh cells. A focus on CXCR5+ and Bcl6^{hi} T cell populations may miss large populations of B cell-helper T cells in target tissues, such as the PD-1^{hi} CXCR5⁺ Tph cell population in RA synovium. A broad assessment of T cells that produce the relevant effector molecules and demonstrate B cell-helper function, aided by high-dimensional analyses such as mass cytometry and RNA-seq, will provide a more complete understanding of the T cell-B cell interactions that promote immune-mediated tissue inflammation and the development of ELS in chronically inflamed tissues.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and approved it for publication.

ACKNOWLEDGMENTS

DR is supported by the Rheumatology Research Foundation Tobe and Stephen E. Malawista, MD Endowment in Academic Rheumatology and by the National Institute of Arthritis and Musculoskeletal and Skin Diseases of the National Institutes of Health under Award Number K08 AR072791-01. The author thanks Michael Brenner and Alexandra Bocharnikov for helpful discussions. The content is solely the responsibility of the author and does not necessarily represent the official views of the National Institutes of Health.

REFERENCES

1. Crotty S. Follicular helper CD4 T cells (TFH). *Ann Rev Immunol.* (2011) 29:621–63. doi: 10.1146/annurev-immunol-031210-101400
2. Forster R, Mattis AE, Kremmer E, Wolf E, Brem G, Lipp M. A putative chemokine receptor, BLR1, directs B cell migration to defined lymphoid organs and specific anatomic compartments of the spleen. *Cell* (1996) 87:1037–47. doi: 10.1016/S0092-8674(00)81798-5
3. Gunn MD, Ngo VN, Ansel KM, Ekland EH, Cyster JG, Williams LT. A B-cell-homing chemokine made in lymphoid follicles activates Burkitt's lymphoma receptor-1. *Nature* (1998) 391:799–803. doi: 10.1038/35876
4. Legler DE, Loetscher M, Roos RS, Clark-Lewis I, Baggiolini M, Moser B. B cell-attracting chemokine 1, a human CXC chemokine expressed in lymphoid tissues, selectively attracts B lymphocytes via BLR1/CXCR5. *J Exp Med.* (1998) 187:655–60. doi: 10.1084/jem.187.4.655
5. Ansel KM, McHeyzer-Williams LJ, Ngo VN, McHeyzer-Williams MG, Cyster JG. *In vivo*-activated CD4 T cells upregulate CXC chemokine receptor 5 and reprogram their response to lymphoid chemokines. *J Exp Med.* (1999) 190:1123–34. doi: 10.1084/jem.190.8.1123
6. Breitfeld D, Ohl L, Kremmer E, Ellwart J, Sallusto F, Lipp M, et al. Follicular B helper T cells express CXC chemokine receptor 5, localize to B cell follicles, and support immunoglobulin production. *J Exp Med.* (2000) 192:1545–52. doi: 10.1084/jem.192.11.1545
7. Kim CH, Rott LS, Clark-Lewis I, Campbell DJ, Wu L, Butcher EC. Subspecialization of CXCR5+ T cells: B helper activity is focused in a germinal center-localized subset of CXCR5+ T cells. *J Exp Med.* (2001) 193:1373–81. doi: 10.1084/jem.193.12.1373
8. Schaerli P, Willmann K, Lang AB, Lipp M, Loetscher P, Moser B. CXC chemokine receptor 5 expression defines follicular homing T cells with B cell helper function. *J Exp Med.* (2000) 192:1553–62. doi: 10.1084/jem.192.11.1553
9. Liu X, Chen X, Zhong B, Wang A, Wang X, Chu F, et al. Transcription factor achaete-scute homologue 2 initiates follicular T-helper-cell development. *Nature* (2014) 507:513–8. doi: 10.1038/nature12910
10. Johnston RJ, Poholek AC, DiToro D, Yusuf I, Eto D, Barnett B, et al. Bcl6 and Blimp-1 are reciprocal and antagonistic regulators of T follicular helper cell differentiation. *Science* (2009) 325:1006–10. doi: 10.1126/science.1175870

11. Nurieva RI, Chung Y, Martinez GJ, Yang XO, Tanaka S, Matskevitch TD, et al. Bcl6 mediates the development of T follicular helper cells. *Science* (2009) 325:1001–5. doi: 10.1126/science.1176676
12. Yu D, Rao S, Tsai LM, Lee SK, He Y, Sutcliffe EL, et al. The transcriptional repressor Bcl-6 directs T follicular helper cell lineage commitment. *Immunity* (2009) 31:457–68. doi: 10.1016/j.immuni.2009.07.002
13. Kroenke MA, Eto D, Locci M, Cho M, Davidson T, Haddad EK, et al. Bcl6 and Maf cooperate to instruct human follicular helper CD4 T cell differentiation. *J Immunol.* (2012) 188:3734–44. doi: 10.4049/jimmunol.1103246
14. Hardtke S, Ohl L, Forster R. Balanced expression of CXCR5 and CCR7 on follicular T helper cells determines their transient positioning to lymph node follicles and is essential for efficient B-cell help. *Blood* (2005) 106:1924–31. doi: 10.1182/blood-2004-11-4494
15. Haynes NM, Allen CD, Lesley R, Ansel KM, Killeen N, Cyster JG. Role of CXCR5 and CCR7 in follicular Th cell positioning and appearance of a programmed cell death gene-1-high germinal center-associated subpopulation. *J Immunol.* (2007) 179:5099–108. doi: 10.4049/jimmunol.179.8.5099
16. Xu H, Li X, Liu D, Li J, Zhang X, Chen X, et al. Follicular T-helper cell recruitment governed by bystander B cells and ICOS-driven motility. *Nature* (2013) 496:523–7. doi: 10.1038/nature12058
17. Hatzi K, Nance JP, Kroenke MA, Bothwell M, Haddad EK, Melnick A, et al. BCL6 orchestrates Th cell differentiation via multiple distinct mechanisms. *J Exp Med.* (2015) 212:539–53. doi: 10.1084/jem.20141380
18. Rasheed AU, Rahn HP, Sallusto F, Lipp M, Muller G. Follicular B helper T cell activity is confined to CXCR5(hi)ICOS(hi) CD4 T cells and is independent of CD57 expression. *Eur J Immunol.* (2006) 36:1892–903. doi: 10.1002/eji.200636136
19. Chevalier N, Jarrossay D, Ho E, Avery DT, Ma CS, Yu D, et al. CXCR5 expressing human central memory CD4 T cells and their relevance for humoral immune responses. *J Immunol.* (2011) 186:5556–68. doi: 10.4049/jimmunol.1002828
20. Chtanova T, Tangye SG, Newton R, Frank N, Hodge MR, Rolph MS, et al. T follicular helper cells express a distinctive transcriptional profile, reflecting their role as non-Th1/Th2 effector cells that provide help for B cells. *J Immunol.* (2004) 173:68–78. doi: 10.4049/jimmunol.173.1.68
21. Zotos D, Coquet JM, Zhang Y, Light A, D'Costa K, Kallies A, et al. IL-21 regulates germinal center B cell differentiation and proliferation through a B cell-intrinsic mechanism. *J Exp Med.* (2010) 207:365–78. doi: 10.1084/jem.20091777
22. Spolski R, Leonard WJ. Interleukin-21: basic biology and implications for cancer and autoimmunity. *Ann Rev Immunol.* (2008) 26:57–79. doi: 10.1146/annurev.immunol.26.021607.090316
23. Benteibibel SE, Schmitt N, Banchereau J, Ueno H. Human tonsil B-cell lymphoma 6 (BCL6)-expressing CD4+ T-cell subset specialized for B-cell help outside germinal centers. *Proc Natl Acad Sci USA.* (2011) 108:E488–97. doi: 10.1073/pnas.1100898108
24. Campbell DJ, Kim CH, Butcher EC. Separable effector T cell populations specialized for B cell help or tissue inflammation. *Nat Immunol.* (2001) 2:876–81. doi: 10.1038/ni0901-876
25. Mueller SN, Gebhardt T, Carbone FR, Heath WR. Memory T cell subsets, migration patterns, and tissue residence. *Ann Rev Immunol.* (2013) 31:137–61. doi: 10.1146/annurev-immunol-032712-095954
26. Pitzalis C, Jones GW, Bombardieri M, Jones SA. Ectopic lymphoid-like structures in infection, cancer and autoimmunity. *Nat Rev.* (2014) 14:447–62. doi: 10.1038/nri3700
27. Takemura S, Braun A, Crowson C, Kurtin PJ, Cofield RH, O'Fallon WM, et al. Lymphoid neogenesis in rheumatoid synovitis. *J Immunol.* (2001) 167:1072–80. doi: 10.4049/jimmunol.167.2.1072
28. Scheel T, Gursche A, Zacher J, Haupl T, Berek C. V-region gene analysis of locally defined synovial B and plasma cells reveals selected B cell expansion and accumulation of plasma cell clones in rheumatoid arthritis. *Arthr Rheumat.* (2011) 63:63–72. doi: 10.1002/art.27767
29. Humby F, Bombardieri M, Manzo A, Kelly S, Blades MC, Kirkham B, et al. Ectopic lymphoid structures support ongoing production of class-switched autoantibodies in rheumatoid synovium. *PLoS Med.* (2009) 6:e1. doi: 10.1371/journal.pmed.0060001
30. Chang A, Henderson SG, Brandt D, Liu N, Guttikonda R, Hsieh C, et al. *In situ* B cell-mediated immune responses and tubulointerstitial inflammation in human lupus nephritis. *J Immunol.* (2011) 186:1849–60. doi: 10.4049/jimmunol.1001983
31. Cipponi A, Mercier M, Seremet T, Baurain JF, Theate I, van den Oord J, et al. Neogenesis of lymphoid structures and antibody responses occur in human melanoma metastases. *Cancer Res.* (2012) 72:3997–4007. doi: 10.1158/0008-5472.CAN-12-1377
32. Coronella JA, Spier C, Welch M, Trevor KT, Stopeck AT, Villar H, et al. Antigen-driven oligoclonal expansion of tumor-infiltrating B cells in infiltrating ductal carcinoma of the breast. *J Immunol.* (2002) 169:1829–36. doi: 10.4049/jimmunol.169.4.1829
33. Nzula S, Going JJ, Stott DI. Antigen-driven clonal proliferation, somatic hypermutation, and selection of B lymphocytes infiltrating human ductal breast carcinomas. *Cancer Res.* (2003) 63:3275–80. Available online at: <http://cancerres.aacrjournals.org/content/63/12/3275.long>
34. Ferdman J, Porcheray F, Gao B, Moore C, DeVito J, Dougherty S, et al. Expansion and somatic hypermutation of B-cell clones in rejected human kidney grafts. *Transplantation* (2014) 98:766–72. doi: 10.1097/TP.000000000000124
35. Sprent J, Surh CD. T cell memory. *Ann Rev Immunol.* (2002) 20:551–79. doi: 10.1146/annurev.immunol.20.100101.151926
36. Weisel F, Shlomchik M. Memory B cells of mice and humans. *Ann Rev Immunol.* (2017) 35:255–84. doi: 10.1146/annurev-immunol-041015-055531
37. Liarski VM, Kaverina N, Chang A, Brandt D, Yanez D, Talasnik L, et al. Cell distance mapping identifies functional T follicular helper cells in inflamed human renal tissue. *Sci Trans Med.* (2014) 6:230ra46. doi: 10.1126/scitranslmed.3008146
38. Cohen-Kaminsky S, Ranchoux B, Perros F. CXCL13 in tertiary lymphoid tissues: sites of production are different from sites of functional localization. *Am J Respir Crit Care Med.* (2014) 189:369–70. doi: 10.1164/rccm.201307-1389LE
39. Litsiou E, Semitekolou M, Galani IE, Morianos I, Tsoutsas A, Kara P, et al. CXCL13 production in B cells via Toll-like receptor/lymphotoxin receptor signaling is involved in lymphoid neogenesis in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med.* (2013) 187:1194–202. doi: 10.1164/rccm.201208-1543OC
40. Yang BG, Tanaka T, Jang MH, Bai Z, Hayasaka H, Miyasaka M. Binding of lymphoid chemokines to collagen IV that accumulates in the basal lamina of high endothelial venules: its implications in lymphocyte trafficking. *J Immunol.* (2007) 179:4376–82. doi: 10.4049/jimmunol.179.7.4376
41. Orr C, Najm A, Biniecka M, McGarry T, Ng CT, Young F, et al. Synovial immunophenotype and anti-citrullinated peptide antibodies in rheumatoid arthritis patients: relationship to treatment response and radiologic prognosis. *Arthr Rheumatol.* (2017) 69:2114–23. doi: 10.1002/art.40218
42. Manzo A, Bombardieri M, Humby F, Pitzalis C. Secondary and ectopic lymphoid tissue responses in rheumatoid arthritis: from inflammation to autoimmunity and tissue damage/remodeling. *Immunol Rev.* (2010) 233:267–85. doi: 10.1111/j.0105-2896.2009.00861.x
43. Pitzalis C, Kelly S, Humby F. New learnings on the pathophysiology of RA from synovial biopsies. *Curr Opin Rheumatol.* (2013) 25:334–44. doi: 10.1097/BOR.0b013e32835fd8eb
44. Berek C, Kim HJ. B-cell activation and development within chronically inflamed synovium in rheumatoid and reactive arthritis. *Sem Immunol.* (1997) 9:261–8. doi: 10.1006/smim.1997.0076
45. Nanki T, Takada K, Komano Y, Morio T, Kanegane H, Nakajima A, et al. Chemokine receptor expression and functional effects of chemokines on B cells: implication in the pathogenesis of rheumatoid arthritis. *Arthr Res Ther.* (2009) 11:R149. doi: 10.1186/ar2823
46. Rosengren S, Wei N, Kalunian KC, Zvaifler NJ, Kavanaugh A, Boyle DL. Elevated autoantibody content in rheumatoid arthritis synovium with lymphoid aggregates and the effect of rituximab. *Arthr Res Ther.* (2008) 10:R105. doi: 10.1186/ar2497
47. Kraan MC, Haringman JJ, Post WJ, Versendaal J, Breedveld FC, Tak PP. Immunohistological analysis of synovial tissue for differential diagnosis in early arthritis. *Rheumatology* (1999) 38:1074–80. doi: 10.1093/rheumatology/38.11.1074

48. Orange DE, Agius P, DiCarlo EF, Robine N, Geiger H, Szymonifka J, et al. Identification of three rheumatoid arthritis disease subtypes by machine learning integration of synovial histologic features and RNA Sequencing Data. *Arthr Rheumatol.* (2018) 70:690–701. doi: 10.1002/art.40428
49. Donlin LT, Rao DA, Wei K, Slowikowski K, McGeachy MJ, Turner JD, et al. Methods for high-dimensional analysis of cells dissociated from cryopreserved synovial tissue. *Arthritis Res Ther.* (2018) 20:139. doi: 10.1186/s13075-018-1631-y
50. Rao DA, Gurish MF, Marshall JL, Slowikowski K, Fonseka CY, Liu Y, et al. Pathologically expanded peripheral T helper cell subset drives B cells in rheumatoid arthritis. *Nature* (2017) 542:110–4. doi: 10.1038/nature20810
51. Koch AE, Kunkel SL, Harlow LA, Johnson B, Evanoff HL, Haines GK, et al. Enhanced production of monocyte chemoattractant protein-1 in rheumatoid arthritis. *J Clin Invest.* (1992) 90:772–9. doi: 10.1172/JCI115950
52. Katschke KJ Jr, Rottman JB, Ruth JH, Qin S, Wu L, LaRosa G, et al. Differential expression of chemokine receptors on peripheral blood, synovial fluid, and synovial tissue monocytes/macrophages in rheumatoid arthritis. *Arthr Rheumat.* (2001) 44:1022–32. doi: 10.1002/1529-0131(200105)44:5<1022::AID-ANR181>3.0.CO;2-N
53. Ruth JH, Rottman JB, Katschke KJ Jr, Qin S, Wu L, LaRosa G, et al. Selective lymphocyte chemokine receptor expression in the rheumatoid joint. *Arthr Rheumat.* (2001) 44:2750–60. doi: 10.1002/1529-0131(200112)44:12<2750::AID-ART462>3.0.CO;2-C
54. Shadidi KR, Aarvak T, Henriksen JE, Natvig JB, Thompson KM. The chemokines CCL5, CCL2 and CXCL12 play significant roles in the migration of Th1 cells into rheumatoid synovial tissue. *Scand J Immunol.* (2003) 57:192–8. doi: 10.1046/j.1365-3083.2003.01214.x
55. Haringman JJ, Smeets TJ, Reinders-Blankert P, Tak PP. Chemokine and chemokine receptor expression in paired peripheral blood mononuclear cells and synovial tissue of patients with rheumatoid arthritis, osteoarthritis, and reactive arthritis. *Ann Rheum Dis.* (2006) 65:294–300. doi: 10.1136/ard.2005.037176
56. Vergunst CE, Gerlag DM, Lopatinskaya L, Klareskog L, Smith MD, van den Bosch F, et al. Modulation of CCR2 in rheumatoid arthritis: a double-blind, randomized, placebo-controlled clinical trial. *Arthr Rheumat.* (2008) 58:1931–9. doi: 10.1002/art.23591
57. van Kuijk AW, Vergunst CE, Gerlag DM, Bresnihan B, Gomez-Reino JJ, Rouzier R, et al. CCR5 blockade in rheumatoid arthritis: a randomised, double-blind, placebo-controlled clinical trial. *Ann Rheum Dis.* (2010) 69:2013–6. doi: 10.1136/ard.2010.131235
58. Lebre MC, Vergunst CE, Choi IY, Aarass S, Oliveira AS, Wyant T, et al. Why CCR2 and CCR5 blockade failed and why CCR1 blockade might still be effective in the treatment of rheumatoid arthritis. *PLoS ONE* (2011) 6:e21772. doi: 10.1371/journal.pone.0021772
59. Leong YA, Chen Y, Ong HS, Wu D, Man K, Deleage C, et al. CXCR5(+) follicular cytotoxic T cells control viral infection in B cell follicles. *Nat Immunol.* (2016) 17:1187–96. doi: 10.1038/ni.3543
60. Thauinat O, Field AC, Dai J, Louedec L, Patey N, Bloch MF, et al. Lymphoid neogenesis in chronic rejection: evidence for a local humoral alloimmune response. *Proc Natl Acad Sci USA.* (2005) 102:14723–8. doi: 10.1073/pnas.0507223102
61. Huibers MM, Gareau AJ, Beerthuijzen JM, Siera-de Koning E, van Kuik J, Kamburova EG, et al. Donor-specific antibodies are produced locally in ectopic lymphoid structures in cardiac allografts. *Am J Transpl.* (2017) 17:246–54. doi: 10.1111/ajt.13969
62. Deteix C, Attuill-Audenis V, Duthey A, Patey N, McGregor B, Dubois V, et al. Intra-graft Th17 infiltrate promotes lymphoid neogenesis and hastens clinical chronic rejection. *J Immunol.* (2010) 184:5344–51. doi: 10.4049/jimmunol.0902999
63. de Graaf GN, Dieterich M, Hesselink DA, Boer K, Clahsen-van Groningen MC, Kraaijeveld R, et al. Follicular T helper cells and humoral reactivity in kidney transplant patients. *Clin Exp Immunol.* (2015) 180:329–40. doi: 10.1111/cei.12576
64. de Leur K, Clahsen-van Groningen MC, van den Bosch TPP, de Graaf GN, Hesselink DA, Samsom JN, et al. Characterization of ectopic lymphoid structures in different types of acute renal allograft rejection. *Clin Exp Immunol.* (2018) 192:224–32. doi: 10.1111/cei.13099
65. Magliozzi R, Howell O, Vora A, Serafini B, Nicholas R, Puopolo M, et al. Meningeal B-cell follicles in secondary progressive multiple sclerosis associate with early onset of disease and severe cortical pathology. *Brain* (2007) 130:1089–104. doi: 10.1093/brain/awm038
66. Lovato L, Willis SN, Rodig SJ, Caron T, Almendinger SE, Howell OW, et al. Related B cell clones populate the meninges and parenchyma of patients with multiple sclerosis. *Brain* (2011) 134:534–41. doi: 10.1093/brain/awq350
67. Tzartos JS, Craner MJ, Friese MA, Jakobsen KB, Newcombe J, Esiri MM, et al. IL-21 and IL-21 receptor expression in lymphocytes and neurons in multiple sclerosis brain. *Am J Pathol.* (2011) 178:794–802. doi: 10.1016/j.ajpath.2010.10.043
68. Wan Y, Bai Y, Sun S, Qiu X, Zheng Y, Wang H, et al. Increased expression of transcription factor Bcl-6 in chronic rhinosinusitis with nasal polyps. *Eur Arch Oto-rhino-Laryngol.* (2016) 273:391–9. doi: 10.1007/s00405-015-3498-8
69. Xiao L, Jia L, Zhang Y, Yu S, Wu X, Yang B, et al. Human IL-21+IFN- γ CD4+ T cells in nasal polyps are regulated by IL-12. *Sci Rep.* (2015) 5:12781. doi: 10.1038/srep12781
70. Zhang YN, Song J, Wang H, Wang H, Zeng M, Zhai GT, et al. Nasal IL-4⁺CXCR5⁺CD4⁺ T follicular helper cell counts correlate with local IgE production in eosinophilic nasal polyps. *J Allergy Clin Immunol.* (2016) 137:462–73. doi: 10.1016/j.jaci.2015.07.025
71. Stone JH, Zen Y, Deshpande V. IgG4-related disease. *N Eng J Med.* (2012) 366:539–51. doi: 10.1056/NEJMra1104650
72. Maehara T, Moriyama M, Nakashima H, Miyake K, Hayashida JN, Tanaka A, et al. Interleukin-21 contributes to germinal centre formation and immunoglobulin G4 production in IgG4-related dacryoadenitis and sialoadenitis, so-called Mikulicz's disease. *Ann Rheum Dis.* (2012) 71:2011–19. doi: 10.1136/annrheumdis-2012-201477
73. Kamekura R, Takano K, Yamamoto M, Kawata K, Shigehara K, Jitsukawa S, et al. Cutting edge: a critical role of lesional T follicular helper cells in the pathogenesis of IgG4-related disease. *J Immunol.* (2017) 199:2624–9. doi: 10.4049/jimmunol.1601507
74. Le Pottier L, Devauchelle V, Fautrel A, Daridon C, Saraux A, Youinou P, et al. Ectopic germinal centers are rare in Sjogren's syndrome salivary glands and do not exclude autoreactive B cells. *J Immunol.* (2009) 182:3540–7. doi: 10.4049/jimmunol.0803588
75. Odegard JM, Marks BR, DiPlacido LD, Poholek AC, Kono DH, Dong C, et al. ICOS-dependent extrafollicular helper T cells elicit IgG production via IL-21 in systemic autoimmunity. *J Exp Med.* (2008) 205:2873–86. doi: 10.1084/jem.20080840
76. Lee SK, Rigby RJ, Zotos D, Tsai LM, Kawamoto S, Marshall JL, et al. B cell priming for extrafollicular antibody responses requires Bcl-6 expression by T cells. *J Exp Med.* (2011) 208:1377–88. doi: 10.1084/jem.20102065
77. Poholek AC, Hansen K, Hernandez SG, Eto D, Chande A, Weinstein JS, et al. *In vivo* regulation of Bcl6 and T follicular helper cell development. *J Immunol.* (2010) 185:313–26. doi: 10.4049/jimmunol.0904023
78. Vu Van D, Beier KC, Pietzke LJ, Al Baz MS, Feist RK, Gurka S, et al. Local T/B cooperation in inflamed tissues is supported by T follicular helper-like cells. *Nat Commun.* (2016) 7:10875. doi: 10.1038/ncomms10875
79. Coquet JM, Schuijs MJ, Smyth MJ, Deswarte K, Beyaert R, Braun H, et al. Interleukin-21-producing CD4(+) T cells promote type 2 immunity to house dust mites. *Immunity* (2015) 43:318–30. doi: 10.1016/j.immuni.2015.07.015
80. Astorri E, Bombardieri M, Gabba S, Peakman M, Pozzilli P, Pitzalis C. Evolution of ectopic lymphoid neogenesis and *in situ* autoantibody production in autoimmune nonobese diabetic mice: cellular and molecular characterization of tertiary lymphoid structures in pancreatic islets. *J Immunol.* (2010) 185:3359–68. doi: 10.4049/jimmunol.1001836
81. Papadakis KA, Prehn J, Nelson V, Cheng L, Binder SW, Ponath PD, et al. The role of thymus-expressed chemokine and its receptor CCR9 on lymphocytes in the regional specialization of the mucosal immune system. *J Immunol.* (2000) 165:5069–76. doi: 10.4049/jimmunol.165.9.5069
82. McGuire HM, Vogelzang A, Ma CS, Hughes WE, Silveira PA, Tangye SG, et al. A subset of interleukin-21+ chemokine receptor CCR9+ T helper cells target accessory organs of the digestive system in autoimmunity. *Immunity* (2011) 34:602–15. doi: 10.1016/j.immuni.2011.01.021
83. Blokland LM, Hillen MR, Kruize AA, Meller S, Homey B, Smithson GM, et al. Increased CCL25 and T helper cells expressing CCR9 in the salivary

- glands of patients with primary sjogren's syndrome: potential new axis in lymphoid neogenesis. *Arthr Rheumatol.* (2017) 69:2038–51. doi: 10.1002/art.40182
84. Haskett S, Ding J, Zhang W, Thai A, Cullen P, Xu S, et al. Identification of novel CD4+ T cell subsets in the target tissue of sjogren's syndrome and their differential regulation by the lymphotoxin/LIGHT signaling axis. *J Immunol.* (2016) 197:3806–19. doi: 10.4049/jimmunol.1600407
 85. Miyauchi K, Sugimoto-Ishige A, Harada Y, Adachi Y, Usami Y, Kaji T, et al. Protective neutralizing influenza antibody response in the absence of T follicular helper cells. *Nat Immunol.* (2016) 17:1447–58. doi: 10.1038/ni.3563
 86. Carpio VH, Opata MM, Montanez ME, Banerjee PP, Dent AL, Stephens R. IFN-gamma and IL-21 double producing T cells are Bcl6-independent and survive into the memory phase in plasmodium chabaudi infection. *PLoS ONE* (2015) 10:e0144654. doi: 10.1371/journal.pone.0144654
 87. Lu KT, Kanno Y, Cannons JL, Handon R, Bible P, Elkahoul AG, et al. Functional and epigenetic studies reveal multistep differentiation and plasticity of *in vitro*-generated and *in vivo*-derived follicular T helper cells. *Immunity* (2011) 35:622–32. doi: 10.1016/j.immuni.2011.07.015
 88. Nurieva RI, Chung Y, Hwang D, Yang XO, Kang HS, Ma L, et al. Generation of T follicular helper cells is mediated by interleukin-21 but independent of T helper 1, 2, or 17 cell lineages. *Immunity* (2008) 29:138–49. doi: 10.1016/j.immuni.2008.05.009
 89. Suto A, Kashiwakuma D, Kagami S, Hirose K, Watanabe N, Yokote K, et al. Development and characterization of IL-21-producing CD4+ T cells. *J Exp Med.* (2008) 205:1369–79. doi: 10.1084/jem.20072057
 90. Ma CS, Suryani S, Avery DT, Chan A, Nanan R, Santner-Nanan B, et al. Early commitment of naive human CD4(+) T cells to the T follicular helper (TFH) cell lineage is induced by IL-12. *Immunol Cell Biol.* (2009) 87:590–600. doi: 10.1038/icb.2009.64
 91. Schmitt N, Liu Y, Bentebibel SE, Munagala I, Bourdery L, Venuprasad K, et al. The cytokine TGF-beta co-opts signaling via STAT3-STAT4 to promote the differentiation of human TFH cells. *Nat Immunol.* (2014) 15:856–65. doi: 10.1038/ni.2947
 92. Nakayama S, Kanno Y, Takahashi H, Jankovic D, Lu KT, Johnson TA, et al. Early Th1 cell differentiation is marked by a Tfh cell-like transition. *Immunity* (2011) 35:919–31. doi: 10.1016/j.immuni.2011.11.012
 93. Hiramatsu Y, Suto A, Kashiwakuma D, Kanari H, Kagami S, Ikeda K, et al. c-Maf activates the promoter and enhancer of the IL-21 gene, and TGF-beta inhibits c-Maf-induced IL-21 production in CD4+ T cells. *J Leukocyte Biol.* (2010) 87:703–12. doi: 10.1189/jlb.0909639
 94. Pot C, Jin H, Awasthi A, Liu SM, Lai CY, Madan R, et al. Cutting edge: IL-27 induces the transcription factor c-Maf, cytokine IL-21, and the costimulatory receptor ICOS that coordinately act together to promote differentiation of IL-10-producing Tr1 cells. *J Immunol.* (2009) 183:797–801. doi: 10.4049/jimmunol.0901233
 95. Bauquet AT, Jin H, Paterson AM, Mitsdoerffer M, Ho IC, Sharpe AH, et al. The costimulatory molecule ICOS regulates the expression of c-Maf and IL-21 in the development of follicular T helper cells and TH-17 cells. *Nat Immunol.* (2009) 10:167–75. doi: 10.1038/ni.1690
 96. Ansel KM, Ngo VN, Hyman PL, Luther SA, Forster R, Sedgwick JD, et al. A chemokine-driven positive feedback loop organizes lymphoid follicles. *Nature* (2000) 406:309–14. doi: 10.1038/35018581
 97. Luther SA, Lopez T, Bai W, Hanahan D, Cyster JG. BCL6 expression in pancreatic islets causes B cell recruitment and lymphotoxin-dependent lymphoid neogenesis. *Immunity* (2000) 12:471–81. doi: 10.1016/S1074-7613(00)80199-5
 98. Zheng B, Ozen Z, Zhang X, De Silva S, Marinova E, Guo L, et al. CXCL13 neutralization reduces the severity of collagen-induced arthritis. *Arthr Rheum.* (2005) 52:620–6. doi: 10.1002/art.20768
 99. Bracke KR, Verhamme FM, Seys LJ, Bantsimba-Malanda C, Cunoosamy DM, Herbst R, et al. Role of CXCL13 in cigarette smoke-induced lymphoid follicle formation and chronic obstructive pulmonary disease. *Am J Respir Crit Care Med.* (2013) 188:343–55. doi: 10.1164/rccm.201211-2055OC
 100. Brodfuehrer J, Rankin A, Edmonds J, Keegan S, Andreyeva T, Lawrence-Henderson R, et al. Quantitative analysis of target coverage and germinal center response by a CXCL13 neutralizing antibody in a T-dependent mouse immunization model. *Pharm Res.* (2014) 31:635–48. doi: 10.1007/s11095-013-1185-2
 101. Yamamoto K, Nishiumi S, Yang L, Klimatcheva E, Pandina T, Takahashi S, et al. Anti-CXCL13 antibody can inhibit the formation of gastric lymphoid follicles induced by Helicobacter infection. *Mucos Immunol.* (2014) 7:1244–54. doi: 10.1038/mi.2014.14
 102. Wu X, Guo J, Ding R, Lv B, Bi L. CXCL13 blockade attenuates lupus nephritis of MRL/lpr mice. *Acta Histochem.* (2015) 117:732–7. doi: 10.1016/j.acthis.2015.09.001
 103. Sharma A, Kiripolsky J, Klimatcheva E, Howell A, Fereidouni F, Levenson R, et al. Early BAFF receptor blockade mitigates murine Sjogren's syndrome: concomitant targeting of CXCL13 and the BAFF receptor prevents salivary hypofunction. *Clin Immunol.* (2016) 164:85–94. doi: 10.1016/j.clim.2016.01.015
 104. Yu H, Shahsafaei A, Dorfman DM. Germinal-center T-helper-cell markers PD-1 and CXCL13 are both expressed by neoplastic cells in angioimmunoblastic T-cell lymphoma. *Am J Clin Pathol.* (2009) 131:33–41. doi: 10.1309/AJCP62WRKERXPXDRT
 105. Gu-Trantien C, Migliori E, Buisseret L, de Wind A, Brohee S, Garaud S, et al. CXCL13-producing TFH cells link immune suppression and adaptive memory in human breast cancer. *JCI Insight* (2017) 2:e91487. doi: 10.1172/jci.insight.91487
 106. Havenar-Daughton C, Lindqvist M, Heit A, Wu JE, Reiss SM, Kendric K, et al. CXCL13 is a plasma biomarker of germinal center activity. *Proc Natl Acad Sci USA.* (2016) 113:2702–7. doi: 10.1073/pnas.1520112113
 107. Manzo A, Vitolo B, Humby F, Caporali R, Jarrossay D, Dell'Accio F, et al. Mature antigen-experienced T helper cells synthesize and secrete the B cell chemoattractant CXCL13 in the inflammatory environment of the rheumatoid joint. *Arthr Rheumat.* (2008) 58:3377–87. doi: 10.1002/art.23966
 108. Kobayashi S, Murata K, Shibuya H, Morita M, Ishikawa M, Furu M, et al. A distinct human CD4+ T cell subset that secretes CXCL13 in rheumatoid synovium. *Arthr Rheumat.* (2013) 65:3063–72. doi: 10.1002/art.38173
 109. Stephenson W, Donlin LT, Butler A, Rozo C, Bracken B, Rashidfarrokhi A, et al. Single-cell RNA-seq of rheumatoid arthritis synovial tissue using low-cost microfluidic instrumentation. *Nat Commun.* (2018) 9:791. doi: 10.1038/s41467-017-02659-x
 110. Dennis G Jr., Holweg CT, Kummerfeld SK, Choy DF, Setiadi AF, Hackney JA, et al. Synovial phenotypes in rheumatoid arthritis correlate with response to biologic therapeutics. *Arthr Res Ther.* (2014) 16:R90. doi: 10.1186/ar4555
 111. Bugatti S, Manzo A, Vitolo B, Benaglio F, Binda E, Scarabelli M, Humby F, et al. High expression levels of the B cell chemoattractant CXCL13 in rheumatoid synovium are a marker of severe disease. *Rheumatology* (2014) 53:1886–95. doi: 10.1093/rheumatology/keu163
 112. Ngo VN, Korner H, Gunn MD, Schmidt KN, Riminton DS, Cooper MD, et al. Lymphotoxin alpha/beta and tumor necrosis factor are required for stromal cell expression of homing chemokines in B and T cell areas of the spleen. *J Exp Med.* (1999) 189:403–12. doi: 10.1084/jem.189.2.403
 113. Rangel-Moreno J, Carragher DM, de la Luz Garcia-Hernandez M, Hwang JY, Kusser K, Hartson L, et al. The development of inducible bronchus-associated lymphoid tissue depends on IL-17. *Nat Immunol.* (2011) 12:639–46. doi: 10.1038/ni.2053
 114. Zheng C, Zheng L, Yoo JK, Guo H, Zhang Y, Guo X, et al. Landscape of infiltrating T cells in liver cancer revealed by single-cell sequencing. *Cell* (2017) 169:1342–56 e16. doi: 10.1016/j.cell.2017.05.035
 115. Chen MM, Xiao X, Lao XM, Wei Y, Liu RX, Zeng QH, et al. Polarization of tissue-resident TFH-like cells in human hepatoma bridges innate monocyte inflammation and M2b macrophage polarization. *Cancer Discov.* (2016) 6:1182–95. doi: 10.1158/2159-8290.CD-16-0329
 116. Gu-Trantien C, Loi S, Garaud S, Equeter C, Libin M, de Wind A, et al. CD4(+) follicular helper T cell infiltration predicts breast cancer survival. *J Clin Invest.* (2013) 123:2873–92. doi: 10.1172/JCI67428
 117. Workel HH, Lubbers JM, Arnold R, van der Vlies P, de Lange K, Bosse T, van Gool I, et al. TGF-β induced CXCL13 in CD8+ T cells is associated with tertiary lymphoid structures in cancer. *bioRxiv [Preprint]* (2018) doi: 10.1101/303834

118. Bindea G, Mlecnik B, Tosolini M, Kirilovsky A, Waldner M, Obenauf AC, et al. Spatiotemporal dynamics of intratumoral immune cells reveal the immune landscape in human cancer. *Immunity* (2013) 39:782–95. doi: 10.1016/j.immuni.2013.10.003
119. Wei SC, Levine JH, Cogdill AP, Zhao Y, Anang NAS, Andrews MC, et al. Distinct cellular mechanisms underlie anti-CTLA-4 and anti-PD-1 checkpoint blockade. *Cell* (2017) 170:1120–33 e17. doi: 10.1016/j.cell.2017.07.024
120. Zappasodi R, Budhu S, Hellmann MD, Postow MA, Senbabaoglu Y, Manne S, et al. Non-conventional inhibitory CD4(+)Foxp3(-)PD-1(hi) T cells as a biomarker of immune checkpoint blockade activity. *Cancer Cell* (2018) 33:1017–32 e7.
121. Kobayashi S, Watanabe T, Suzuki R, Furu M, Ito H, Ito J, et al. TGF-beta induces the differentiation of human CXCL13-producing CD4(+) T cells. *Eur J Immunol.* (2016) 46:360–71. doi: 10.1002/eji.201546043
122. Locci M, Wu JE, Arumemi F, Mikulski Z, Dahlberg C, Miller AT, et al. Activin A programs the differentiation of human TFH cells. *Nat Immunol.* (2016) 17:976–84. doi: 10.1038/ni.3494
123. Takagi R, Higashi T, Hashimoto K, Nakano K, Mizuno Y, Okazaki Y, et al. B cell chemoattractant CXCL13 is preferentially expressed by human Th17 cell clones. *J Immunol.* (2008) 181:186–9. doi: 10.4049/jimmunol.181.1.186
124. Kimura N, Yamagiwa S, Sugano T, Setsu T, Tominaga K, Kamimura H, et al. Possible involvement of chemokine C-C receptor 7(-) programmed cell death-1(+) follicular helper T-cell subset in the pathogenesis of autoimmune hepatitis. *J Gastroenterol Hepatol.* (2018) 33:298–306. doi: 10.1111/jgh.13844
125. Taylor DK, Mittereder N, Kuta E, Delaney T, Burwell T, Dacosta K, et al. T follicular helper-like cells contribute to skin fibrosis. *Sci Trans Med.* (2018) 10. doi: 10.1126/scitranslmed.aaf5307
126. Wang L, Sun Y, Zhang Z, Jia Y, Zou Z, Ding J, et al. CXCR5+ CD4+ T follicular helper cells participate in the pathogenesis of primary biliary cirrhosis. *Hepatology* (2015) 61:627–38. doi: 10.1002/hep.27306
127. Zhu C, Ma J, Liu Y, Tong J, Tian J, Chen J, et al. Increased frequency of follicular helper T cells in patients with autoimmune thyroid disease. *J Clin Endocrinol Metab.* (2012) 97:943–50. doi: 10.1210/jc.2011-2003

Conflict of Interest Statement: DR is an inventor in a patent submitted on Tph cells.

Copyright © 2018 Rao. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



Low Peripheral T Follicular Helper Cells in Perinatally HIV-Infected Children Correlate With Advancing HIV Disease

Bret McCarty¹, Mussa Mwamzuka², Fatma Marshed², Matthew Generoso¹, Patricia Alvarez¹, Tiina Ilmet¹, Adam Kravietz¹, Aabid Ahmed², William Borkowsky¹, Derya Unutmaz³ and Alka Khaitan^{1,4*}

¹ Department of Pediatrics, Division of Infectious Diseases, New York University School of Medicine, New York, NY, United States, ² Bomu Hospital, Mombasa, Kenya, ³ Jackson Laboratory for Genomic Medicine, Farmington, CT, United States, ⁴ Department of Microbiology, New York University School of Medicine, New York, NY, United States

OPEN ACCESS

Edited by:

Shahram Salek-Ardakani,
Pfizer, United States

Reviewed by:

Ramon Arens,
Leiden University Medical Center,
Netherlands
Savita Pahwa,
University of Miami, United States
Georges Abboud,
University of Florida, United States

*Correspondence:

Alka Khaitan
alka.khaitan@nyumc.org

Specialty section:

This article was submitted to
T Cell Biology,
a section of the journal
Frontiers in Immunology

Received: 03 May 2018

Accepted: 01 August 2018

Published: 24 August 2018

Citation:

McCarty B, Mwamzuka M, Marshed F, Generoso M, Alvarez P, Ilmet T, Kravietz A, Ahmed A, Borkowsky W, Unutmaz D and Khaitan A (2018) Low Peripheral T Follicular Helper Cells in Perinatally HIV-Infected Children Correlate With Advancing HIV Disease. *Front. Immunol.* 9:1901. doi: 10.3389/fimmu.2018.01901

Background: T follicular helper (Tfh) cells are crucial for B cell differentiation and antigen-specific antibody production. Dysregulation of Tfh-mediated B cell help weakens B cell responses in HIV infection. Moreover, Tfh cells in the lymph node and peripheral blood comprise a significant portion of the latent HIV reservoir. There is limited data on the effects of perinatal HIV infection on Tfh cells in children. We examined peripheral Tfh (pTfh) cell frequencies and phenotype in HIV-infected children and their associations with disease progression, immune activation, and B cell differentiation.

Methods: In a Kenyan cohort of 76 perinatally HIV-infected children, comprised of 43 treatment-naïve (ART-) and 33 on antiretroviral therapy (ART+), and 42 healthy controls (HIV-), we identified memory pTfh cells, T cell activation markers, and B cell differentiation states using multi-parameter flow cytometry. Soluble CD163 and intestinal fatty acid-binding protein plasma levels were quantified by ELISA.

Results: ART- children had reduced levels of pTfh cells compared with HIV- children that increased with antiretroviral therapy. HIV+ children had higher programmed cell death protein 1 (PD-1) expression on pTfh cells, regardless of treatment status. Low memory pTfh cells with elevated PD-1 levels correlated with advancing HIV disease status, indicated by increasing HIV viral loads and T cell and monocyte activation, and decreasing %CD4 and CD4:CD8 ratios. Antiretroviral treatment, particularly when started at younger ages, restored pTfh cell frequency and eliminated correlations with disease progression, but failed to lower PD-1 levels on pTfh cells and their associations with CD4 T cell percentages and activation. Altered B cell subsets, with decreased naïve and resting memory B cells and increased activated and tissue-like memory B cells in HIV+ children, correlated with low memory pTfh cell frequencies. Last, HIV+ children had decreased proportions of CXCR5+ CD8 T cells that associated with low %CD4 and CD4:CD8 ratios.

Conclusion: Low memory pTfh cell frequencies with high PD-1 expression in HIV+ children correlate with worsening disease status and an activated and differentiated B cell profile. This perturbed memory pTfh cell population may contribute to weak vaccine and HIV-specific antibody responses in HIV+ children. Restoring Tfh cell capacity may be important for novel pediatric HIV cure and vaccine strategies.

Keywords: T follicular helper cells, HIV, children, immune activation, B cells, T follicular cytotoxic cells

INTRODUCTION

T follicular helper (Tfh) cells are a recently described CD4 T cell subset that links the adaptive and humoral immune systems. These cells are identified by expression of chemokine receptor CXCR5 that directs their migration to B cell follicles in response to CXCL13. Once localized in B cell follicles, Tfh cells form germinal centers (GCs) (1). Their differentiation and functions are regulated by B-cell lymphoma 6 (Bcl-6) (2). Tfh cells stimulate B cell differentiation to plasma and memory B cells (3) and are critical for antigen-specific antibody production, class switching, and B cell memory differentiation (4). During natural infections or after vaccinations, Tfh cell interactions with B cells mediate high affinity class-switched antibody production and B cell memory development (3). Tfh cells exert effector functions by secretion of IL-21 in addition to small levels of Th1 and Th2 cytokines IFN γ and IL-4 (4, 5). Dysfunctional Tfh cells can result in autoantibodies and have been associated with autoimmune diseases such as rheumatoid arthritis and systemic lupus erythematosus (6–10).

A small portion of CD4 T cells closely resembling tissue-resident Tfh cells are found in the peripheral blood (10–12). These peripheral Tfh (pTfh) cells also provide B cell help, but require secondary signals that include CD40L and inducible T-cell costimulator (ICOS) interactions and IL-21 secretion from B cells (11). Phenotypically, pTfh cells differ from lymphoid Tfh cells. Bcl-6 is downregulated in circulating CXCR5+ CD4 T cells, and thus fails to identify pTfh cells (10, 11). Second, while programmed cell death protein 1 (PD-1) is constitutively expressed on lymphoid Tfh cells, in the periphery, PD-1 is variably expressed, with low levels on resting pTfh cells and high levels on activated pTfh cells (12–14).

T follicular helper cells are critical for clearance of acute and chronic viral infections and effective virus-specific antibody production. In HIV vaccine trials, improved humoral responses occurred in subjects with expanded HIV-specific IL-21+ pTfh cells (14). Moreover, certain Tfh cell subsets correlate with the development of broadly neutralizing HIV antibodies (13, 15). Indeed, Tfh cells are being investigated for novel HIV vaccine strategies (11, 13, 15). Studies of HIV-infected adults demonstrate that circulating Tfh cells are decreased while lymphoid Tfh cells are paradoxically expanded, functionally impaired, and preferentially infected with replication-competent HIV (14, 16–20). Most importantly, Tfh cells in peripheral blood and lymph nodes (LNs) comprise a major compartment of the latent HIV reservoir (14, 21). Thus, Tfh cells may have both

beneficial and pathologic roles during HIV infection—they are critical for HIV-specific humoral responses yet are also selective targets of HIV infection and enable HIV persistence in latent reservoirs.

Both lymphoid and circulating Tfh cells have impaired function in HIV+ adults, which may contribute to weakened responses to vaccines (16, 20, 22, 23). The potential consequences of muted antibody responses are magnified in children with perinatal HIV infection during routine childhood vaccinations. However, few studies have examined pTfh cells in children (18–20, 24). We evaluated pTfh cell frequencies and phenotype in children with perinatal HIV infection and their associations with HIV disease progression and B cell subsets. We found decreased pTfh cell levels in untreated HIV+ children compared with HIV negative children, which failed to normalize within 1 year of antiretroviral treatment. pTfh cells had elevated PD-1 expression in HIV+ children regardless of treatment status. Low pTfh cell frequencies with high PD-1 expression correlated with HIV disease progression and an activate/differentiated B cell distribution. Finally, CXCR5+ memory CD8 T cells were depleted in HIV+ children.

MATERIALS AND METHODS

Participants

Ethical approval for this study was obtained from New York University (10-02586) and Kenyatta National Hospital/University of Nairobi (P283/07/2011). Written informed consent and verbal assent when appropriate were obtained from all participants and/or parents. We enrolled a total of 76 perinatally infected HIV+ and 42 HIV negative-unexposed children (HIV–) aged 5–18 years old from Bomu Hospital in Mombasa, Kenya between 2011 and 2012. HIV+ children included 43 antiretroviral therapy naïve (ART–) and 33 HIV+ children on antiretroviral treatment for at least 6 months with viral load less than or equal to 5,000 copies/mL (ART+). Treatment timing and duration for ART+ subjects is shown in Table S1 in Supplementary Material. Individuals with a recent acute illness, active *Mycobacterium tuberculosis* or malaria infection, or pregnancy within one year were ineligible for study entry.

Plasma and peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood by centrifugation and Ficoll-Hypaque (GE Healthcare) density gradient centrifugation then cryopreserved in –80°C and liquid nitrogen, respectively. HIV RNA PCR was performed on diluted plasma samples with Roche,

TABLE 1 | Subject characteristics.

	HIV–	ART–	ART+	<i>p</i>
<i>n</i>	42	43	33	
Age (years) ^d	11 (9–13)	11 (8–14)	12 (8–13)	NS ^a
Female	17 (41%)	23 (54%)	19 (58%)	NS ^b
%CD4 ^d	38 (33–42)	24 (13–28)	32 (27–41)	<i>p</i> < 0.0001 ^a
Absolute CD4 count ^d	923 (739–1,247)	583 (352–812)	962 (750–1,228)	<i>p</i> < 0.0001 ^a
HIV log copies/mL ^d		4.8 (4.2–5.2)	2 (2–2)	<i>p</i> < 0.0001 ^c

^aKruskal–Wallis test.^bChi squared test.^cMann–Whitney test.^dMedian values with upper and lower quartile range.

COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test, version 2.0 (limit of detection 110 copies/mL).

HIV–, ART–, and ART+ were matched for age and sex (Table 1). Median CD4% in HIV– children was 38 (IQR 33–42). ART– had median CD4% of 24 (IQR 13–28) and HIV viral load of 4.8 (IQR 4.2–5.2) log copies/mL. ART+ had median CD4% and HIV viral load of 32 (IQR 27–41) and 2 (IQR 2–2) log copies/mL, respectively (Table 1).

Flow Cytometric Studies

Cryopreserved PBMCs were evaluated by flow cytometry with fluorescent-conjugated antibodies to CD3, CD4, CD8, CD45RO, CCR7, CXCR5, PD-1, CD38, HLA-DR, CD19, CD21, CD27, and β 7. Cells were stained at 4°C for 30 min in PBS buffer containing 2% FCS and 0.1% sodium azide. Stained cells were analyzed using LSRII flow cytometer (BD Bioscience) and Flow Jo software (Tree Star). Singlet lymphocytes were gated based on forward and side scatter properties.

Plasma sCD163 and Intestinal Fatty Acid-Binding Protein (I-FABP)

Plasma levels of sCD163 and I-FABP were quantified by ELISA assay using Human sCD163 and I-FABP DuoSet kits (R&D Systems) per the manufacturer's instructions. Plasma samples were diluted 1:100 for sCD163 and 1:1,500 for I-FABP assays based on plasma titration studies to achieve levels within the range of the standard curve concentrations provided in the commercial ELISA kit according to the manufacturer's recommendation. Each test was performed in duplicate with results reported as the average of duplicate results.

Statistics

All statistical analyses were performed using GraphPad Prism software. For comparison of multiple groups of subjects, the Kruskal–Wallis test was performed, followed by the two-stage linear step-up procedure of Benjamini, Krieger, and Yekutieli to correct for multiple comparisons by controlling the false discovery rate. Multiple time points were evaluated with Wilcoxon matched-pairs signed-rank test. Correlations were assessed with the Spearman rank test. Threshold of significance for all tests was less than 0.05.

RESULTS

Memory pTfh Cells Are Decreased in Untreated HIV+ Children and Correlate With Disease Progression

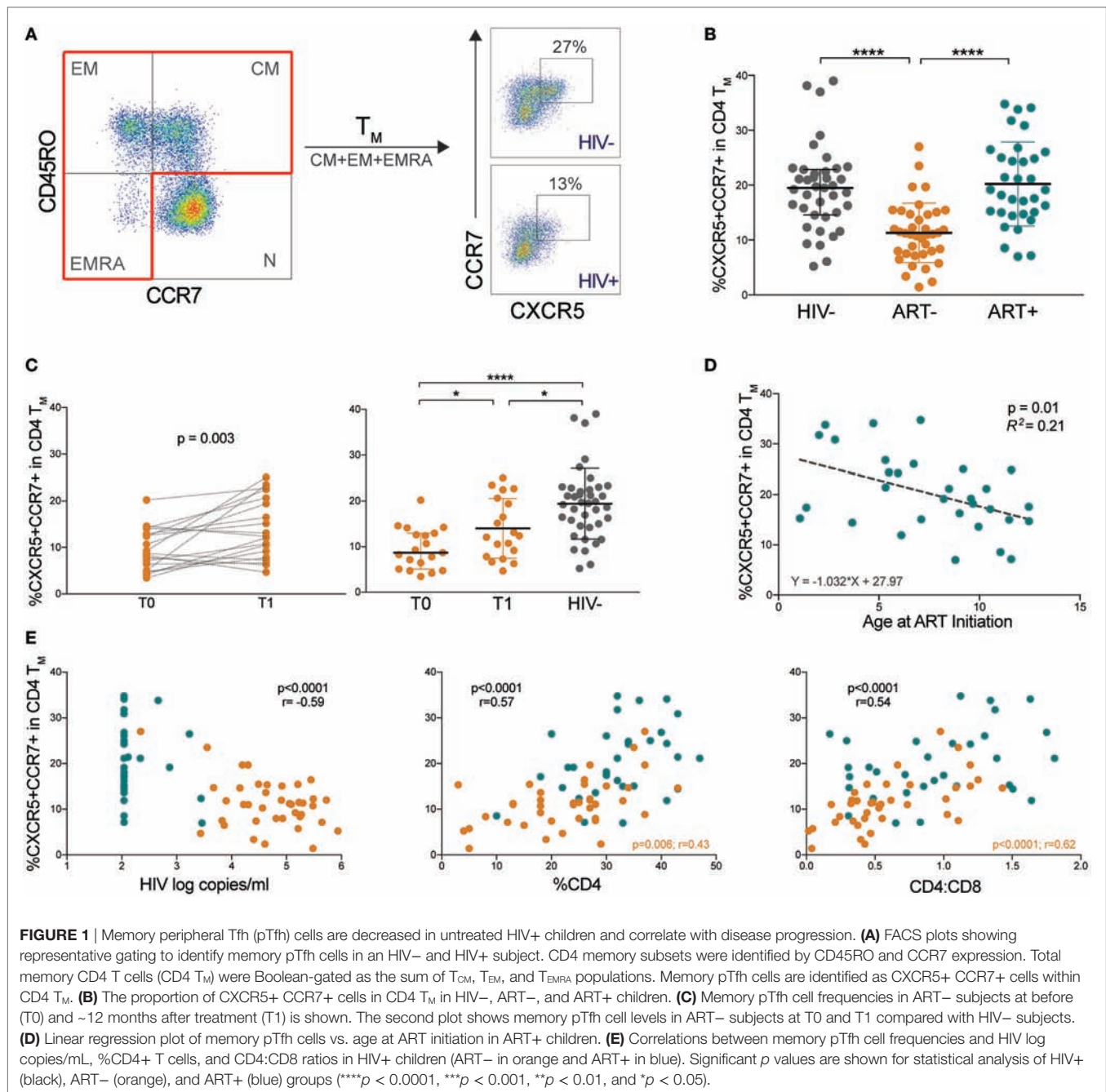
We identified pTfh cells by CXCR5 co-expressed with LN homing receptor CCR7 within memory CD4 T cells as described (25). Memory CD4 T cells (T_M) were identified as the sum of central (T_{CM} , CD45RO+CCR7+), effector (T_{EM} , CD45RO+ CCR7–), and RA+ effector (T_{EMRA} , CD45RO–CCR7–) subsets (Figure 1A). CXCR5+CCR7+ CD4 T_M (memory pTfh) levels were lower in ART– children compared with HIV– and ART+ (*p* < 0.0001; Figure 1B), even when adjusted for age as a potential confounder (Table S2 in Supplementary Material).

In a subset of ART– children who began antiretroviral therapy memory pTfh cell frequencies increased after ~12 months of treatment (*p* = 0.003; Figure 1C). However, these levels remained lower than HIV– children (*p* = 0.02; Figure 1C). In ART+ children, treatment initiation at an earlier age predicted preserved CXCR5+CCR7+ CD4 T_M (*p* = 0.01, R^2 = 0.21; Figure 1D). To determine the clinical relevance of decreased pTfh cell frequencies, we examined correlations between memory pTfh cells and clinical markers of HIV disease progression. In HIV+ subjects, memory pTfh cell levels correlated inversely with HIV viral load (*p* < 0.0001, *r* = –0.59) and directly with %CD4 (*p* < 0.0001, *r* = 0.57) and CD4:CD8 ratios (*p* < 0.0001, *r* = 0.54; Figure 1E). In separate analyses of ART– and ART+ children, memory pTfh frequencies correlated with %CD4 (*p* = 0.006, *r* = 0.43) and CD4:CD8 ratios (*p* < 0.0001, *r* = 0.62) in ART– but not in ART+ children. These correlations were not present in HIV– subjects (Figure S1A in Supplementary Material).

Low Memory pTfh Cell Frequencies Correlate With Immune Activation and Gut Mucosal Disruption in HIV+ Children

We next determined whether memory pTfh cell levels correlated with immune activation markers CD38 and HLA-DR, which are strong predictors of HIV disease progression (26). Memory pTfh cell frequencies inversely correlated with CD38+HLA-DR+ CD4 (*p* < 0.0001, *r* = –0.62) in HIV+ children and in separate analysis of ART– (*p* = 0.003, *r* = –0.47) and ART+ subjects (*p* = 0.04, *r* = –0.37; Figure 2A). Memory pTfh cell frequencies also negatively correlated with CD38+HLA-DR+ CD8 T cells (*p* = 0.006, *r* = –0.32) and monocyte activation, measured by plasma sCD163 concentrations in HIV+ children (*p* < 0.0001, *r* = –0.48), but not when divided into ART– and ART+ groups (Figures 2A,B). There were no significant correlations between pTfh cell frequencies and T cell or monocyte activation in HIV– children (Figures S1B,C in Supplementary Material).

Chronic inflammation in HIV is driven largely by a compromise of the gut mucosa, where the majority of CD4 T cells reside (27). In mouse models, Tfh cells have been shown to play a unique role in maintaining healthy gut homeostasis (28). To study a potential relationship between memory pTfh cells and the gut mucosa in humans, we evaluated correlations with two key gut-related proteins: β 7 integrin and I-FABP. β 7 is a subunit of

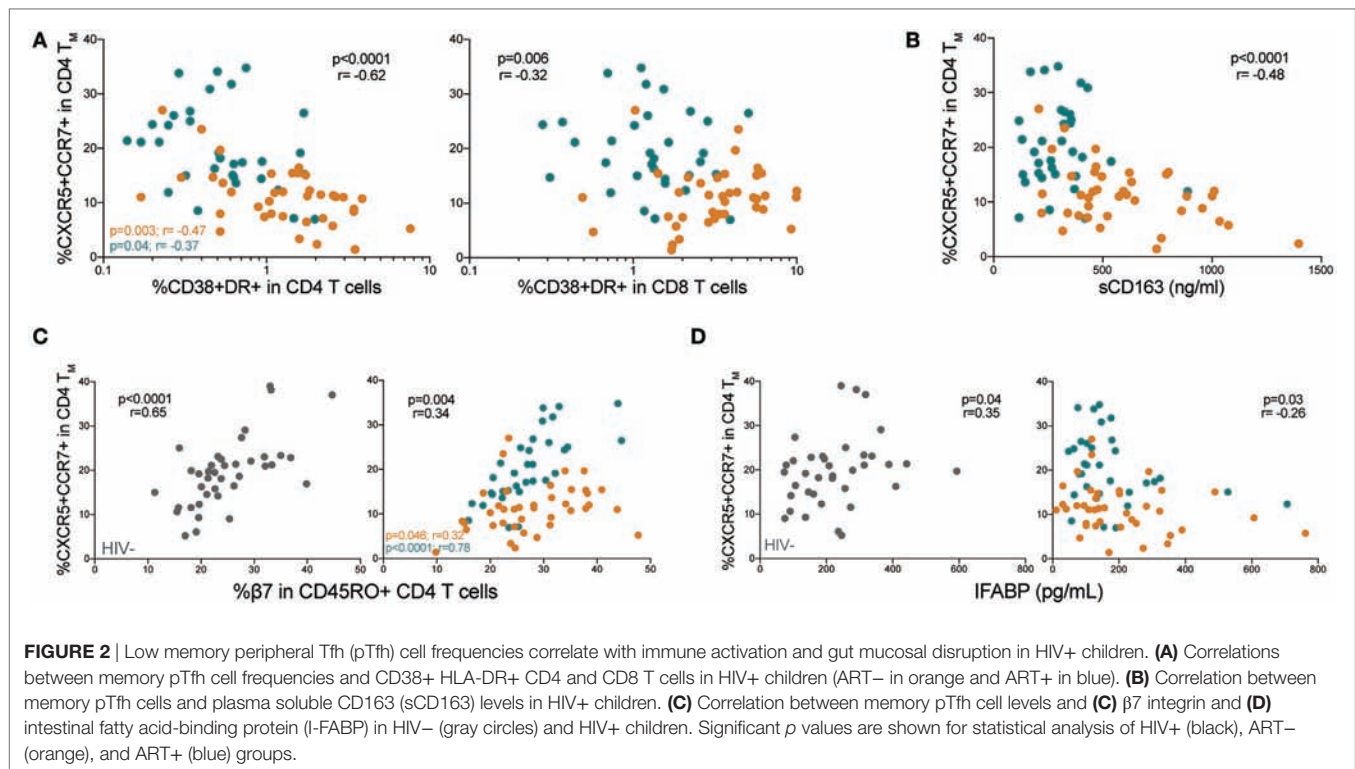


the gut-homing receptor and HIV co-receptor $\alpha_4\beta_7$ (29). I-FABP is expressed in epithelial cells of the small intestine and is released into the circulation following intestinal mucosal damage (30). Memory pTfh cell levels directly correlated with β_7 +CD45RO+ CD4 T cell frequencies, in both HIV- ($p < 0.0001$, $r = 0.65$) and HIV+ children ($p = 0.004$, $r = 0.34$; **Figure 2C**). ART+ children had a stronger correlation between pTfh cell frequency and β_7 expression in memory CD4 T cells ($p < 0.0001$, $r = 0.78$) compared with ART- children ($p = 0.046$, $r = 0.32$; **Figure 2C**). There was a direct correlation between memory pTfh cell frequencies and I-FABP levels ($p = 0.04$, $r = 0.35$) in HIV- children, and

an indirect correlation in HIV+ children ($p = 0.03$, $r = -0.26$; **Figure 2D**) but not separately in ART- and ART+ groups.

Memory pTfh Cells in HIV+ Children Express High PD-1 Levels That Correlate With Disease Progression

We next evaluated PD-1 expression on memory pTfh cells (gating strategy shown in Figure S2A in Supplementary Material). Both ART- and ART+ subjects had higher PD-1 expression on memory pTfh cells compared with HIV- children ($p < 0.0001$



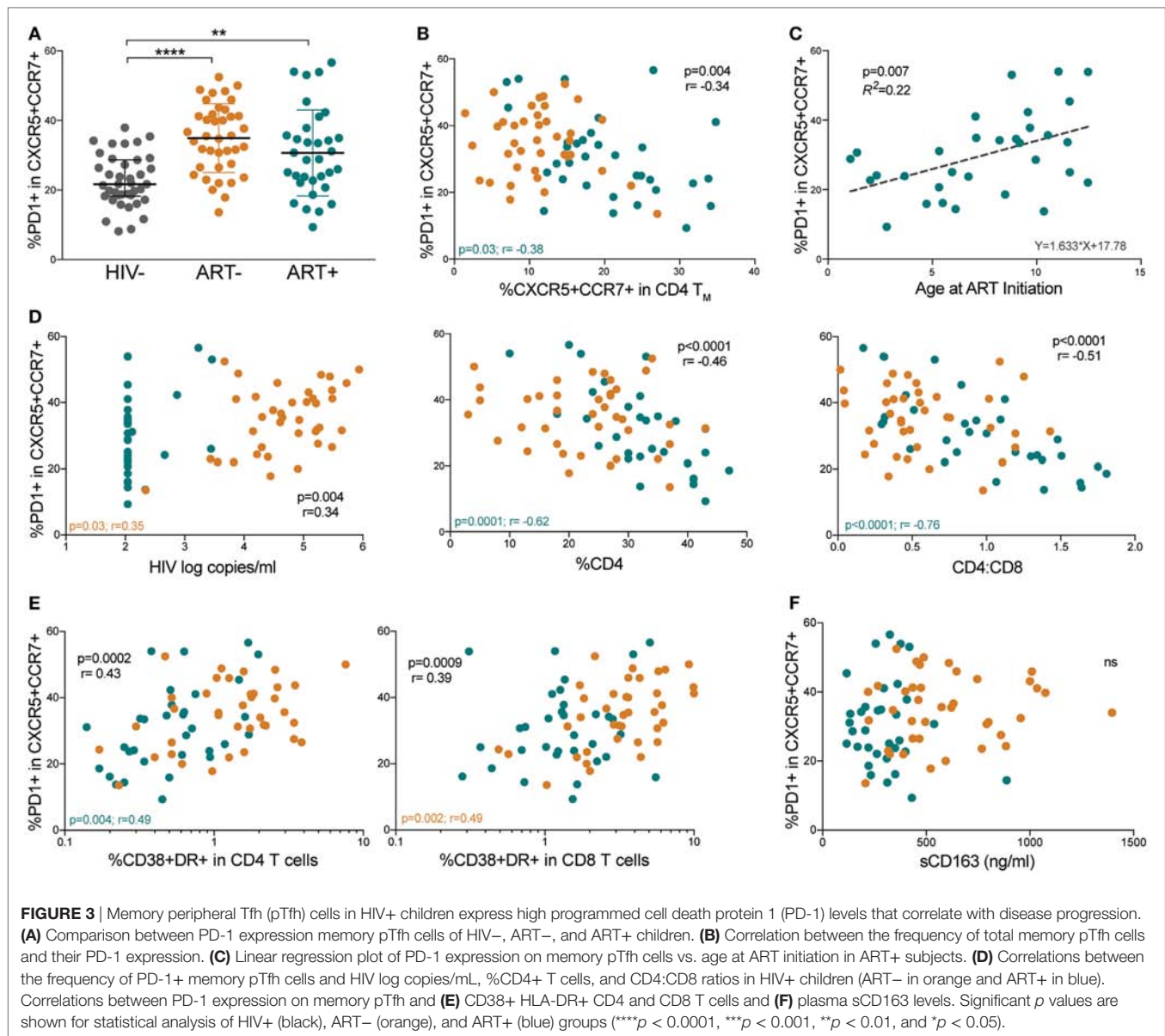
and $p = 0.003$, respectively; **Figure 3A**). PD-1 levels on memory pTfh cells negatively correlated with pTfh cell frequency in HIV+ ($p = 0.004$, $r = -0.34$) and ART+ children ($p = 0.03$, $r = -0.38$) but not in ART- children (**Figure 3B**). In ART+ children, earlier age at ART initiation predicted lower PD-1 expression on memory pTfh cells ($p = 0.007$, $R^2 = 0.22$; **Figure 3C**). PD-1+ memory pTfh cell frequencies correlated directly with HIV viral load ($p = 0.004$, $r = 0.34$), and inversely with %CD4 ($p < 0.0001$, $r = -0.51$; **Figure 3D**) but not in HIV- children (Figure S2B in Supplementary Material). PD-1+ expression on memory pTfh cell correlated directly with CD38+HLA-DR+ CD4 ($p = 0.0002$, $r = 0.43$) and CD8 T cells ($p = 0.0009$, $r = 0.39$; **Figure 3E**) but did not correlate with plasma sCD163 levels (**Figure 3F**) in HIV+ children. In ART- children, PD-1 expression on pTfh cells significantly correlated with HIV viral load ($p = 0.03$, $r = 0.35$, **Figure 2D**) and CD38+HLA-DR+ CD8 T cells ($p = 0.002$, $r = 0.49$; **Figure 3E**). In ART+ children, PD-1 expression correlated inversely with %CD4 ($p = 0.0001$, $r = -0.62$) and CD4:CD8 ratios ($p < 0.0001$, $r = -0.76$; **Figure 3D**). There were no significant correlations between PD-1 expression on pTfh cells and %CD4 or immune activation markers in HIV- children (Figures S2C,D in Supplementary Material).

Differentiated B Cell Populations Correlate With Low Memory pTfh Cells Expressing High PD-1 Levels

Because Tfh cells are intricately linked to B cell differentiation, we evaluated B cell populations and their associations with memory

pTfh cell frequencies and PD-1 expression. Total B cell frequencies were decreased in both ART- and ART+ compared with HIV- children ($p = 0.005$ and $p = 0.02$, respectively; **Figure 4A**). IgD expression on B cells was increased in ART- and ART+ children compared with HIV- children ($p = 0.002$ and $p = 0.0001$, respectively; **Figure 4B**), indicating muted class switching. We further sub-classified B cells into differentiation states of naïve mature (B_N , CD21+CD27-), resting memory (B_{RM} , CD21+CD27+), activated memory (B_{AM} , CD21-CD27+), and exhausted/tissue-like memory (B_{TLM} , CD21-CD27-; **Figure 4C**) subsets. ART+ had increased B_N frequencies compared with HIV- and ART- ($p < 0.0001$; **Figure 4D**). ART- and ART+ had decreased B_{RM} levels compared with HIV- ($p < 0.0001$; **Figure 4E**). B_{RM} levels were higher in ART+ compared with ART- children ($p = 0.002$; **Figure 4E**). ART- had elevated B_{AM} and B_{TLM} cell frequencies compared with HIV- ($p = 0.003$ and $p < 0.0001$) and ART+ ($p < 0.0001$; **Figures 4F,G**). ART+ had lower B_{AM} frequencies compared with HIV- ($p = 0.003$; **Figure 4F**).

We next examined associations between B cell differentiation states and memory pTfh cell frequencies. In HIV+ subjects, memory pTfh cell levels correlated directly with B_N ($p = 0.0001$, $r = 0.44$; **Figure 4D**) and B_{RM} frequencies ($p = 0.0003$, $r = 0.41$; **Figure 4E**), and inversely with B_{AM} ($p = 0.0002$, $r = -0.43$; **Figure 4F**) and B_{TLM} frequencies ($p < 0.0001$, $r = -0.52$; **Figure 4G**). Last, in HIV+ subjects, PD-1 expression on memory pTfh cells correlated inversely with B_N ($p = 0.03$, $r = -0.26$; **Figure 4D**) and B_{RM} ($p = 0.002$, $r = -0.36$; **Figure 4E**), and directly with B_{AM} ($p = 0.046$, $r = 0.24$; **Figure 4F**) and B_{TLM} levels ($p = 0.0006$, $r = 0.40$; **Figure 4G**). These correlations were insignificant when separated into ART- and ART+ subjects except PD-1 expression on pTfh cells in ART- children inversely correlated



with B_N frequency ($p = 0.02$, $r = -0.38$; **Figure 4D**) and directly with B_{TLM} frequency ($p = 0.003$, $r = 0.46$; **Figure 4G**). Memory pTfh cells and their PD-1 expression did not correlate with any B cell subpopulations in HIV- subjects (Figures S3A–D in Supplementary Material).

HIV+ Children Have Low CXCR5+ Memory CD8 T Cells With Elevated PD-1 Expression

Recently, a CD8 T cell counterpart to Tfh cells, defined as follicular cytotoxic T (Tfc) cells, was found to home to B cell follicles *via* a similar CXCR5-dependent mechanism (31). In the B cell follicle, Tfc cells control viral infection by killing infected Tfh cells and B cells (32, 33). We identified pTfc cells by CXCR5 expression in memory CD8 T cells (**Figure 5A**). CXCR5+ CD8 T_M (memory pTfc) cell frequencies were significantly lower in both ART- and

ART+ children compared with HIV- ($p < 0.0001$ and $p = 0.0002$, respectively; **Figure 5B**) even in multivariate analysis adjusting for age (Table S2 in Supplementary Material). In ART- subjects, memory pTfc cell frequencies increased after ~12 months of antiretroviral treatment ($p = 0.004$), but remained significantly lower than HIV- children ($p = 0.03$; **Figure 5C**).

Next, we determined whether memory pTfc cell levels correlated with clinical markers of HIV disease progression and immune activation. In HIV+ subjects, there was no significant correlation between memory pTfc cell frequencies and HIV viral load. Memory pTfc cells directly correlated with %CD4 ($p = 0.0005$, $r = 0.39$) and CD4:CD8 ratios ($p = 0.0001$, $r = 0.43$) in HIV+ subjects even when divided into ART- and ART+ groups (**Figure 5D**). In HIV- subjects, memory pTfc cell frequencies also correlated with CD4 percentages and CD4:CD8 ratios (%CD4: $p = 0.02$, $r = 0.37$; CD4:CD8: $p = 0.02$, $r = 0.37$; **Figure S4A** in

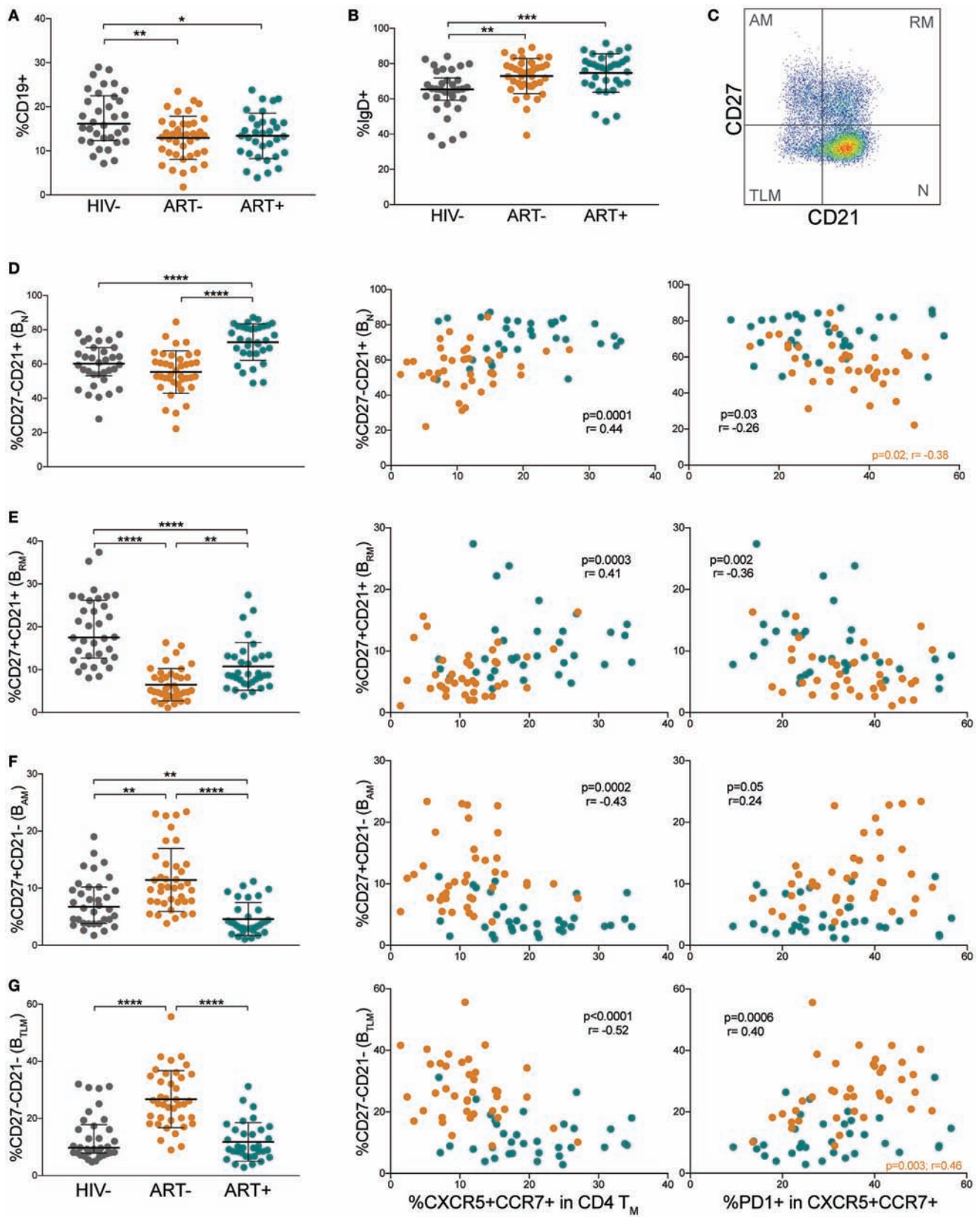


FIGURE 4 | Continued

FIGURE 4 | Differentiated B cell populations correlate with low memory peripheral Tfh (pTfh) cells expressing high programmed cell death protein 1 (PD-1) levels. Comparisons of **(A)** total B cell and **(B)** IgD+ B cell levels in HIV-, ART-, and ART+ children. **(C)** FACS plot showing representative gating to identify B cell differentiation subsets by CD21 and CD27. Plot shown is gated on CD19+ lymphocytes. Comparisons between **(D)** CD27-CD21+ naïve (B_N), **(E)** CD27+ CD21+ resting memory (B_{RM}), **(F)** CD27+ CD21+ activated memory (B_{AM}), and **(G)** CD27-CD21+ tissue-like memory (B_{TLM}) B cell subsets in HIV-, ART-, and ART+ and their correlations with total and PD-1+ memory pTfh cell frequencies in ART- (orange) and ART+ (blue) children. Significant p values are shown for statistical analysis of HIV+ (black), ART- (orange), and ART+ (blue) groups (**** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, and * $p < 0.05$).

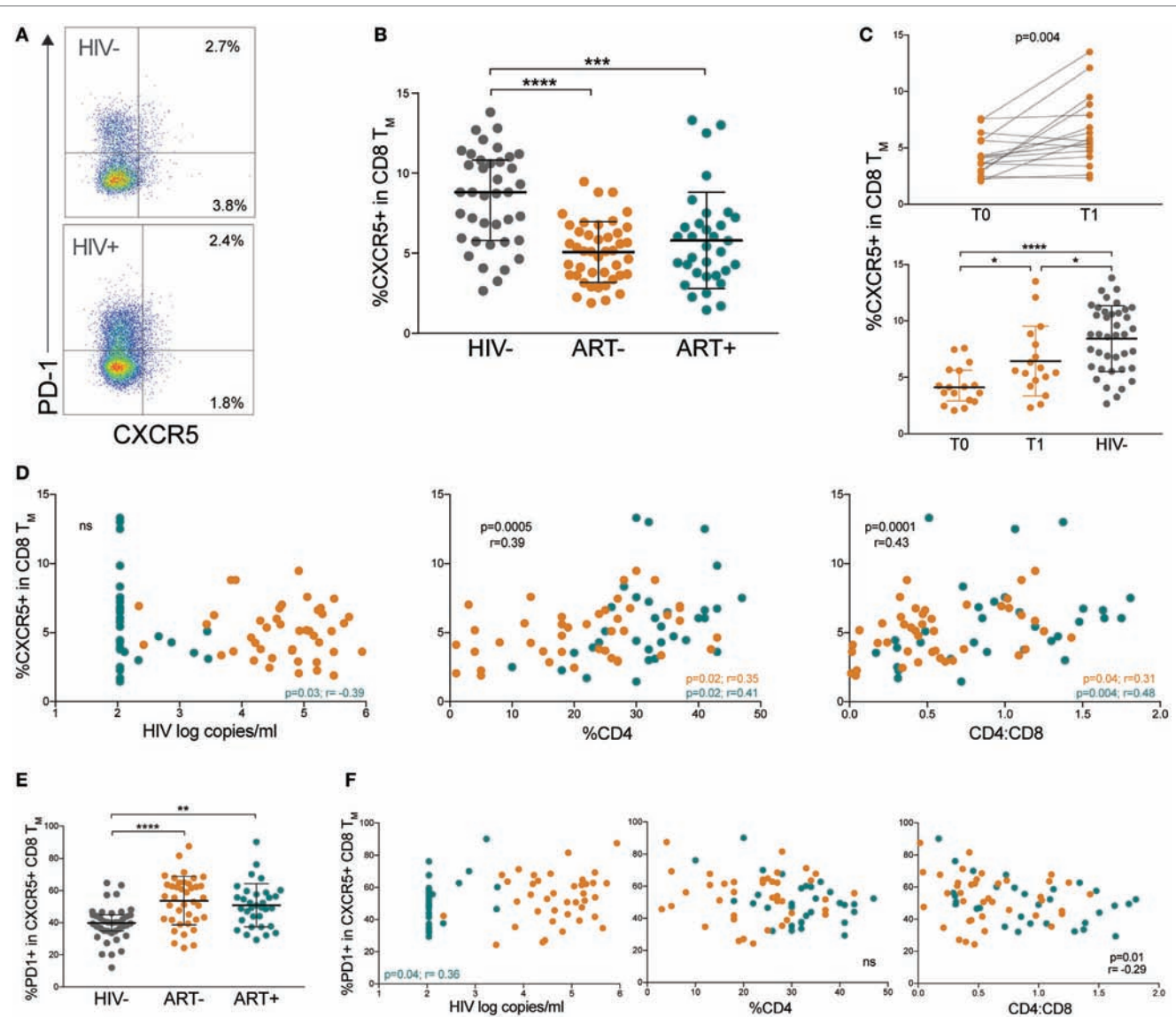


FIGURE 5 | HIV+ children have low CXCR5+ memory CD8 T cells with elevated programmed cell death protein 1 (PD-1). **(A)** FACS plots showing representative gating to identify memory pTfh cells as the total CXCR5+ population in memory CD8 T cells and PD-1 expression in an HIV- and HIV+ subject. Plots shown were gated within total memory CD8 T cells. **(B)** Comparison of CXCR5+ cells within total CD8+ memory T cells in HIV-, ART-, and ART+ children. **(C)** Prospective analysis of memory pTfh in ART- subjects before (T0) and ~12 months after antiretroviral treatment (T1). The second plot compares memory pTfh cell frequencies between ART- subjects at T0 and T1 and HIV- subjects. **(D)** Correlations between memory pTfh cell percentages and HIV log copies/mL, %CD4+ T cells, and CD4:CD8 ratios in HIV+ children (ART- in orange and ART+ in blue). **(E)** PD-1 expression on memory pTfh cells in HIV-, ART-, and ART+ subjects. **(F)** Correlation between PD-1+ memory pTfh cell frequencies and HIV log copies/mL, %CD4+ T cells, and CD4:CD8 ratios in HIV+ children. Significant p values are shown for statistical analysis of HIV+ (black), ART- (orange), and ART+ (blue) groups (**** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, and * $p < 0.05$).

Supplementary Material). We also examined PD-1 expression on memory pTfh cells and found that memory pTfh cells in HIV+ children expressed higher levels of PD-1 compared with HIV– children (ART–: $p < 0.0001$, ART+: $p = 0.001$; **Figure 5E**). In HIV+ children, PD-1 expression on memory pTfh cells inversely correlated with CD4:CD8 ratios ($p = 0.01$, $r = -0.29$), but did not correlate with %CD4 or viral load (**Figure 5F**). In ART+ subjects, HIV viremia correlated negatively with memory pTfh cell frequency ($p = 0.03$, $r = -0.39$; **Figure 1D**) and directly with PD-1 levels on memory pTfh cells ($p = 0.04$, $r = 0.36$; **Figure 5F**). Memory pTfh cell frequencies and their PD-1 expression did not correlate with CD38+HLA-DR+ CD4 or CD8 T cells or sCD163 plasma levels in HIV+ or HIV– children with the exception of a direct correlation between PD-1+CXCR5+ CD8 T_M and CD38+HLA-DR+ CD8 T cells in HIV+ children (Figures S4B,C in Supplementary Material).

DISCUSSION

We demonstrated that untreated children with perinatal HIV infection aged 5–18 years have significantly lower memory pTfh cell frequencies compared with HIV-negative children. Low memory pTfh levels increased with antiretroviral treatment but failed to normalize. In ART+ children, treatment initiation at younger ages predicted preserved pTfh levels. HIV+ children had higher PD-1 expression on pTfh cells regardless of treatment status. Furthermore, lower memory pTfh cells with increased PD-1 expression correlated with worsening HIV disease and an activated and differentiated B cell profile in HIV+ children. Last, HIV+ children have decreased proportions of memory pTfh cells with high PD-1 expression.

In our cohort, low memory pTfh cell frequencies correlated with decreased %CD4 and CD4:CD8 ratios and increased HIV viral load, which may reflect preferential infection of Tfh cells by HIV (14). Muema et al. similarly reported that CXCR5+ CD4 T cells correlated with %CD4 in HIV+ children, yet Bamford et al. failed to find any correlation between pTfh cell frequencies and clinical variables in children. This difference may relate to treatment status, as in our cohort, where these correlations were no longer significant when limited to only ART+ children. While the mechanism of pTfh cell depletion in HIV is not well understood, it is possible that like tissue-resident Tfh cells, pTfh cells are preferentially HIV-infected and killed. It is also plausible that pTfh cells in HIV+ subjects are ill-maintained as a result of impaired crosstalk with B cells, as Tfh and B cells need constant communication *via* co-stimulatory signals to maintain a homeostatic and healthy immune system (2, 16). Alternatively, Tfh cells may be chronically activated and subsequently sequestered in the LNs, depleting levels in the peripheral blood as shown in SIV (34). While Pallikkuth et al. reported a reversible depletion of CXCR5+ cells within T_{CM} in adults with HIV (21), the recovery of pTfh cells with antiretroviral therapy in children is previously unreported. Treatment initiation raised pTfh cell frequencies in ART– subjects, but failed to restore them to normal levels within one year. However, our ART+ subjects had pTfh cell frequencies similar to HIV– children. This may be due to longer duration of antiretroviral treatment or younger age at treatment initiation,

which predicted higher pTfh levels. We speculate that with early antiretroviral treatment, HIV+ children may be able to recover memory pTfh cells, in accordance with the recent recommendation from the World Health Organization to start treatment at the time of HIV diagnosis (35).

Programmed cell death protein 1 is a classic marker of functional Tfh cells in the lymphoid tissue of healthy adults and children, where PD-1/PD-L1 interactions in the GC are crucial for plasma cell differentiation (36). However, there is some uncertainty as to the role of PD-1 on circulating Tfh cells. PD-1 is a co-stimulatory molecule; it is absent on quiescent pTfh cells, expressed on follicular regulatory T (Tfr) cells, and co-expressed with ICOS on activated pTfh cells (37). Interestingly, we demonstrated PD-1 expression on memory pTfh cells was elevated in all HIV+ children regardless of treatment status, and corresponded with worsening HIV disease progression. Moreover, in ART+ children, PD-1 expression correlated with lower CD4 percentages and CD4:CD8 ratios and elevated CD4 T cell activation despite treatment. This indicates that while memory pTfh frequencies recover with antiretrovirals, these cells may still be qualitatively defective, with high PD-1 acting as a potentially pathogenic marker, as has been suggested by previous studies on HIV-specific CD4 (38) and CD8 (39) T cells. Alternatively a portion of PD-1+ pTfh cells may be Tfr cells with suppressive functions that account for low pTfh cell frequencies (40). Elevated PD-1 expression on pTfh cells correlated with increasing CD38+HLA-DR+ CD4 and CD8 T cells, suggesting PD-1+ pTfh cells may be activated Tfh cells associated with inflammation in the adaptive immune system. Interestingly, Pallikkuth et al. reported that CD38+HLA-DR+ expression on pTfh cells decreased after 48 weeks of treatment, but was still significantly higher than healthy controls (21). This activated pTfh cell state likely contributes to their susceptibility as a target for HIV infection and may be linked to high PD-1 expression.

T follicular helper cells also localize in the Peyer's patches of the small intestine. Because there are significant disruptions to the intestinal mucosa during HIV infection, we examined the relationship between Tfh cells and gut-homing receptor $\alpha_4\beta_7$ and I-FABP (41). It was previously shown that I-FABP is increased in perinatally HIV-infected children (42). We found that low memory pTfh cell frequencies in HIV+ subjects correlated with higher plasma I-FABP and lower β_7 + memory CD4 T cell levels. One explanation may be that with worsening gut mucosal disruption, pTfh cells are trafficked to the gut. Localization to the gut may also account for lower circulating pTfh cells during HIV infection. Although we did not co-stain for CXCR5 and β_7 together, pTfh cells likely co-express β_7 to mediate homing to the intestine.

It has been well documented that memory B cell populations and the quality of B cell responses are substantially impaired in HIV+ adults and children (43). More recent reports demonstrate that inadequate B cell help by HIV-infected Tfh cells results in perturbed B cell differentiation and dysregulated antibody production (16, 17). In addition, dysfunctional Tfh cells activate non-specific B cells and lead to hypergammaglobulinemia characteristic of HIV infection (34, 40). Prior groups have shown decreased B_N and B_{RM} cell subsets, as well as expanded B_{AM} and B_{TLM} B cell subsets in HIV+ children (18–20). In our study, we had similar

findings—total B cells were diminished and IgD+ B cells were elevated in HIV-infected children compared with healthy controls. We also demonstrated that B_N were increased in ART+, B_{RM} were low in HIV+, and B_{AM} and B_{TLM} were high in HIV+ compared with healthy controls. While pTfh cell proportions were significantly decreased in the blood, it is possible that with the accumulation of Tfh cells in the LNs, overstimulation by GC Tfh cells in the B cell follicles leads to a shift toward a more differentiated and exhausted B cell state. Moreover, lower total B cells and elevated IgD frequencies in HIV+ children suggest a general insult to the B cell compartment and a defect in Tfh cell function to induce class-switching in memory B cells. Decreased pTfh cells with potentially impaired B cell function may preclude effective HIV antibody responses. Indeed, prior studies report preserved pTfh cells in subjects with broadly neutralizing HIV-specific antibody responses (15, 44). Low pTfh cell frequency was closely linked to a differentiated B cell state in HIV+ children but not when separated into ART– and ART+ groups, suggesting antiretroviral treatment may restore the balance of Tfh cells with B cell differentiation.

Finally, Tfc cells are a novel CD8 T cell subset expressing CXCR5. Tfc cells have been studied in the blood and LN of SIV+ primate models (45, 46), the LNs of LCMV mouse models (33), as well as the blood, tumors, and LN of adult humans (32, 33, 47–49). Multiple groups reported Tfc cells have similar B cell follicle homing abilities to Tfh cells, and the potential to control viral infection by eliminating infected T cells and B cells in the follicle. He et al. reported that HIV-specific Tfc cells were present in the blood of chronically infected adults, and pTfc cells inversely correlated with HIV viral load prior to ART (33). More recently, Jiao et al. reported HIV+ adults had increased CXCR5+ CD8 T cells with high PD-1 expression, which negatively correlated with HIV disease progression (47). To the best of our knowledge, pTfc cell populations in children were not previously studied. HIV+ children had irreversibly depleted CXCR5+ CD8 T_M cells. Notably, antiretroviral treatment raised memory pTfc cell levels but failed to normalize them. As such, pTfc cells directly correlated with %CD4 and CD4:CD8 ratios regardless of treatment status. PD-1 expression was elevated on memory pTfc cells in HIV+ children and negatively correlated with CD4:CD8 ratios and positively associated with CD38+HLA-DR+ CD8 T cells. Interestingly, whereas Jiao et al. concluded CXCR5+ CD8 T cells with high PD-1 expression were highly functional and associated negatively with disease progression, our data demonstrated an association with CD4:CD8 ratios, but not with viremia or CD4 percentages. Our opposing findings may reflect differences in a pediatric cohort or our separate gating strategy on CD8 memory rather than total CD8 T cells.

In conclusion, we demonstrated a marked decrease in peripheral memory Tfh frequencies in untreated children with perinatal HIV infection. These memory pTfh cells increased with antiretroviral therapy but failed to normalize within 1 year. Treatment initiation at younger ages predicted greater recovery of this population. HIV+ children have high PD-1 expression on memory pTfh cells regardless of treatment status. Low memory pTfh cell frequencies with high PD-1 levels correlate with worsening HIV disease status as well as innate and adaptive immune activation. Furthermore, B cell subpopulations are skewed

toward a differentiated and exhausted profile, and coincide with decreased memory pTfh cells in HIV+ children. Finally, memory pTfc cells are depleted in HIV+ children. This perturbed memory pTfh cell population may contribute to weak vaccine and HIV-specific antibody responses in HIV+ children. Together, these findings have important implications for ongoing pediatric HIV cure and vaccine strategies.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of New York University Institutional Review Board and Kenyatta National Hospital/University of Nairobi Ethical Review Committee. The protocol was approved by the New York University Institutional Review Board and Kenyatta National Hospital/University of Nairobi Ethical Review Committee. All subjects gave written informed consent in accordance with the Declaration of Helsinki.

AUTHOR CONTRIBUTIONS

BM analyzed flow cytometry data, drafted the manuscript and figures. MM collected and processed samples and managed data. FM recruited patients and recorded clinical data. AA provided input to study design and oversaw recruitment site. AKravietz and TI performed immune phenotyping studies. MG performed sCD163 ELISA assay. PA performed I-FABP ELISA assay. AKhaitan, WB, and DU conceptualized the study, designed experiments, and interpreted data. AKhaitan supervised the study and edited the manuscript.

ACKNOWLEDGMENTS

We are grateful for all of the children and families who participated in this study. We thank Aparna Alankar for critical reading and valuable suggestions. This study was funded by NIH grant 5K08AI093235-02 to AKhaitan. It was also supported by Centers for Disease Control and Prevention (CDC) Co-operative Agreement (5U2GPS002063-03). The contents are solely the responsibility of the authors and do not necessarily represent the official views of the CDC.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at <https://www.frontiersin.org/articles/10.3389/fimmu.2018.01901/full#supplementary-material>.

FIGURE S1 | Peripheral Tfh (pTfh) cell correlations with %CD4, CD4:CD8 ratios, and immune activation in HIV negative children. Correlations between memory pTfh cells and (A) %CD4 and CD4:CD8 ratios, (B) CD38+ HLA-DR+ CD4 and CD8 T cells, and (C) plasma sCD163 levels in HIV negative children.

FIGURE S2 | PD-1+ memory peripheral Tfh (pTfh) cell gating and correlations in HIV negative children. (A) FACS plots showing representative gating of PD-1+ memory pTfh cells in an HIV– and HIV+ subject. Plots shown were gated within the CXCR5+ CCR7+ CD4+ T_M population. Correlations are shown between PD-1+ memory pTfh cells and (B) %CD4 and CD4:CD8 ratios, (C) CD38+ HLA-DR+ CD4 and CD8 T cells, and (D) plasma sCD163 levels in HIV negative children.

FIGURE S3 | Correlations between memory peripheral Tfh (pTfh) cells and B cell subsets in HIV-negative children. Correlations between total and PD-1+ memory pTfh cells and **(A)** CD27–CD21+ naïve (B_N), **(B)** CD27+ CD21+ resting memory (B_{RM}), **(C)** CD27+ CD21– activated memory (B_{AM}), and **(D)** CD27–CD21– tissue-like memory (B_{TLM}) B cell subsets in HIV negative children.

FIGURE S4 | CXCR5+ memory CD8 T cell subsets and correlations with T cell activation. **(A)** Correlations between CXCR5+ CD8 T_M cell frequencies and %CD4 and CD4:CD8 ratios in HIV– children. Correlations between **(B)** total and **(C)** PD-1+ CXCR5+ CD8 T_M cell frequencies and CD38+ HLA-DR+ CD4 and CD8 T cells and plasma sCD163 levels in HIV– and HIV+ children (ART– in orange and ART+ in blue).

REFERENCES

- Schaerli P, Willmann K, Lang AB, Lipp M, Loetscher P, Moser B. CXC chemokine receptor 5 expression defines follicular homing T cells with B cell helper function. *J Exp Med* (2000) 192(11):1553–62. doi:10.1084/jem.192.11.1553
- Crotty S. Follicular helper CD4 T cells (TFH). *Annu Rev Immunol* (2011) 29:621–63. doi:10.1146/annurev-immunol-031210-101400
- Good-Jacobson KL, Shlomchik MJ. Plasticity and heterogeneity in the generation of memory B cells and long-lived plasma cells: the influence of germinal center interactions and dynamics. *J Immunol* (2010) 185(6):3117–25. doi:10.4049/jimmunol.1001155
- Reinhardt RL, Liang HE, Locksley RM. Cytokine-secreting follicular T cells shape the antibody repertoire. *Nat Immunol* (2009) 10(4):385–93. doi:10.1038/ni.1715
- Yusuf I, Kageyama R, Monticelli L, Johnston RJ, Ditoro D, Hansen K, et al. Germinal center T follicular helper cell IL-4 production is dependent on signaling lymphocytic activation molecule receptor (CD150). *J Immunol* (2010) 185(1):190–202. doi:10.4049/jimmunol.0903505
- Chu Y, Wang F, Zhou M, Chen L, Lu Y. A preliminary study on the characterization of follicular helper T (Tfh) cells in rheumatoid arthritis synovium. *Acta Histochem* (2014) 116(3):539–43. doi:10.1016/j.acthis.2013.10.009
- Ma J, Zhu C, Ma B, Tian J, Baidoo SE, Mao C, et al. Increased frequency of circulating follicular helper T cells in patients with rheumatoid arthritis. *Clin Dev Immunol* (2012) 2012:827480. doi:10.1155/2012/827480
- Wang J, Shan Y, Jiang Z, Feng J, Li C, Ma L, et al. High frequencies of activated B cells and T follicular helper cells are correlated with disease activity in patients with new-onset rheumatoid arthritis. *Clin Exp Immunol* (2013) 174(2):212–20. doi:10.1111/cei.12162
- Le Coz C, Joubin A, Pasquali JL, Korganow AS, Dumortier H, Monneaux F. Circulating TFH subset distribution is strongly affected in lupus patients with an active disease. *PLoS One* (2013) 8(9):e75319. doi:10.1371/journal.pone.0075319
- Simpson N, Gatenby PA, Wilson A, Malik S, Fulcher DA, Tangye SG, et al. Expansion of circulating T cells resembling follicular helper T cells is a fixed phenotype that identifies a subset of severe systemic lupus erythematosus. *Arthritis Rheum* (2010) 62(1):234–44. doi:10.1002/art.25032
- Morita R, Schmitt N, Bentebibel SE, Ranganathan R, Bourdery L, Zurawski G, et al. Human blood CXCR5(+)CD4(+) T cells are counterparts of T follicular cells and contain specific subsets that differentially support antibody secretion. *Immunity* (2011) 34(1):108–21. doi:10.1016/j.immuni.2010.12.012
- He J, Tsai LM, Leong YA, Hu X, Ma CS, Chevalier N, et al. Circulating precursor CCR7(lo)PD-1(hi) CXCR5(+) CD4(+) T cells indicate Tfh cell activity and promote antibody responses upon antigen reexposure. *Immunity* (2013) 39(4):770–81. doi:10.1016/j.immuni.2013.09.007
- Locci M, Havenar-Daughton C, Landais E, Wu J, Kroenke MA, Arlehamn CL, et al. Human circulating PD-1+CXCR3–CXCR5+ memory Tfh cells are highly functional and correlate with broadly neutralizing HIV antibody responses. *Immunity* (2013) 39(4):758–69. doi:10.1016/j.immuni.2013.08.031
- Perreau M, Savoye AL, De Crignis E, Corpataux JM, Cubas R, Haddad EK, et al. Follicular helper T cells serve as the major CD4 T cell compartment for HIV-1 infection, replication, and production. *J Exp Med* (2013) 210(1):143–56. doi:10.1084/jem.20121932
- Cohen K, Altfeld M, Alter G, Stamatatos L. Early preservation of CXCR5+ PD-1+ helper T cells and B cell activation predict the breadth of neutralizing antibody responses in chronic HIV-1 infection. *J Virol* (2014) 88(22):13310–21. doi:10.1128/JVI.02186-14
- Cubas RA, Mudd JC, Savoye AL, Perreau M, van Grevenynghe J, Metcalf T, et al. Inadequate T follicular cell help impairs B cell immunity during HIV infection. *Nat Med* (2013) 19(4):494–9. doi:10.1038/nm.3109
- Lindqvist M, van Lunzen J, Soghoian DZ, Kuhl BD, Ranasinghe S, Kranias G, et al. Expansion of HIV-specific T follicular helper cells in chronic HIV infection. *J Clin Invest* (2012) 122(9):3271–80. doi:10.1172/JCI64314
- Bamford A, Hart M, Lyall H, Goldblatt D, Kelleher P, Kampmann B. The influence of paediatric HIV infection on circulating B cell subsets and CXCR5(+) T helper cells. *Clin Exp Immunol* (2015) 181(1):110–7. doi:10.1111/cei.12618
- Muema DM, Macharia GN, Olusola BA, Hassan AS, Fegan GW, Berkley JA, et al. Proportions of circulating follicular helper T cells are reduced and correlate with memory B cells in HIV-infected children. *PLoS One* (2017) 12(4):e0175570. doi:10.1371/journal.pone.0175570
- Bekele Y, Amu S, Bobosha K, Lantto R, Nilsson A, Endale B, et al. Impaired phenotype and function of T follicular helper cells in HIV-1-infected children receiving ART. *Medicine* (2015) 94(27):e1125. doi:10.1097/MD.0000000000001125
- Pallikkuth S, Sharkey M, Babic DZ, Gupta S, Stone GW, Fischl MA, et al. Peripheral T follicular helper cells are the major HIV reservoir within central memory CD4 T cells in peripheral blood from chronically HIV-infected individuals on combination antiretroviral therapy. *J Virol* (2016) 90(6):2718–28. doi:10.1128/JVI.02883-15
- Boswell KL, Paris R, Boritz E, Ambrozak D, Yamamoto T, Darko S, et al. Loss of circulating CD4 T cells with B cell helper function during chronic HIV infection. *PLoS Pathog* (2014) 10(1):e1003853. doi:10.1371/journal.ppat.1003853
- Pallikkuth S, Parmigiani A, Silva SY, George VK, Fischl M, Pahwa R, et al. Impaired peripheral blood T-follicular helper cell function in HIV-infected nonresponders to the 2009 H1N1/09 vaccine. *Blood* (2012) 120(5):985–93. doi:10.1182/blood-2011-12-396648
- Bekele Y, Yibeltal D, Bobosha K, Andargie TE, Lemma M, Gebre M, et al. T follicular helper cells and antibody response to hepatitis B virus vaccine in HIV-1 infected children receiving ART. *Sci Rep* (2017) 7(1):8956. doi:10.1038/s41598-017-09165-6
- Breitfeld D, Ohl L, Kremmer E, Ellwart J, Sallusto F, Lipp M, et al. Follicular B helper T cells express CXC chemokine receptor 5, localize to B cell follicles, and support immunoglobulin production. *J Exp Med* (2000) 192(11):1545–52. doi:10.1084/jem.192.11.1545
- Giorgi JV, Hultin LE, McKeating JA, Johnson TD, Owens B, Jacobson LP, et al. Shorter survival in advanced human immunodeficiency virus type 1 infection is more closely associated with T lymphocyte activation than with plasma virus burden or virus chemokine coreceptor usage. *J Infect Dis* (1999) 179(4):859–70. doi:10.1086/314660
- Guadalupe M, Reay E, Sankaran S, Prindiville T, Flamm J, McNeil A, et al. Severe CD4+ T-cell depletion in gut lymphoid tissue during primary human immunodeficiency virus type 1 infection and substantial delay in restoration following highly active antiretroviral therapy. *J Virol* (2003) 77(21):11708–17. doi:10.1128/JVI.77.21.11708-11717.2003
- Perruzza L, Gargari G, Proietti M, Fosso B, D'Erchia AM, Faliti CE, et al. T follicular helper cells promote a beneficial gut ecosystem for host metabolic homeostasis by sensing microbiota-derived extracellular ATP. *Cell Rep* (2017) 18(11):2566–75. doi:10.1016/j.celrep.2017.02.061
- Arthos J, Cicala C, Martinelli E, Macleod K, Van Ryk D, Wei D, et al. HIV-1 envelope protein binds to and signals through integrin $\alpha 4 \beta 7$, the gut mucosal homing receptor for peripheral T cells. *Nat Immunol* (2008) 9(3):301–9. doi:10.1038/ni1566
- Lau E, Marques C, Pestana D, Santoalha M, Carvalho D, Freitas P, et al. The role of I-FABP as a biomarker of intestinal barrier dysfunction driven by gut microbiota changes in obesity. *Nutr Metab* (2016) 13:31. doi:10.1186/s12986-016-0089-7
- Quigley ME, Gonzalez VD, Granath A, Andersson J, Sandberg JK. CXCR5+ CCR7– CD8 T cells are early effector memory cells that infiltrate tonsil B cell follicles. *Eur J Immunol* (2007) 37(12):3352–62. doi:10.1002/eji.200636746

32. Leong YA, Chen Y, Ong HS, Wu D, Man K, Deleage C, et al. CXCR5(+) follicular cytotoxic T cells control viral infection in B cell follicles. *Nat Immunol* (2016) 17(10):1187–96. doi:10.1038/ni.3543
33. He R, Hou S, Liu C, Zhang A, Bai Q, Han M, et al. Follicular CXCR5-expressing CD8(+) T cells curtail chronic viral infection. *Nature* (2016) 537(7620):412–28. doi:10.1038/nature19317
34. Petrovas C, Yamamoto T, Gerner MY, Boswell KL, Wloka K, Smith EC, et al. CD4 T follicular helper cell dynamics during SIV infection. *J Clin Invest* (2012) 122(9):3281–94. doi:10.1172/JCI63039
35. WHO. *Consolidated Guidelines on the Use of Antiretroviral Drugs for Treating and Preventing HIV Infection: Recommendations for a Public Health Approach*. 2nd ed. Geneva, Switzerland: WHO (2016).
36. Good-Jacobson KL, Szumilas CG, Chen L, Sharpe AH, Tomayko MM, Shlomchik MJ. PD-1 regulates germinal center B cell survival and the formation and affinity of long-lived plasma cells. *Nat Immunol* (2010) 11:535. doi:10.1038/ni.1877
37. Schmitt N, Ueno H. Blood Tfh cells come with colors. *Immunity* (2013) 39(4):629–30. doi:10.1016/j.immuni.2013.09.011
38. D'Souza M, Fontenot AP, Mack DG, Lozupone C, Dillon S, Meditz A, et al. Programmed death 1 expression on HIV-specific CD4+ T cells is driven by viral replication and associated with T cell dysfunction. *J Immunol* (2007) 179(3):1979–87. doi:10.4049/jimmunol.179.3.1979
39. Trautmann L, Janbazian L, Chomont N, Said EA, Gimmig S, Bessette B, et al. Upregulation of PD-1 expression on HIV-specific CD8+ T cells leads to reversible immune dysfunction. *Nat Med* (2006) 12(10):1198–202. doi:10.1038/nm1106-1329b
40. Chowdhury A, Del Rio Estrada PM, Tharp GK, Tribble RP, Amara RR, Chahroudi A, et al. Decreased T follicular regulatory cell/T follicular helper cell (TFH) in simian immunodeficiency virus-infected rhesus macaques may contribute to accumulation of TFH in chronic infection. *J Immunol* (2015) 195(7):3237–47. doi:10.4049/jimmunol.1402701
41. Kato LM, Kawamoto S, Maruya M, Fagarasan S. Gut TFH and IgA: key players for regulation of bacterial communities and immune homeostasis. *Immunol Cell Biol* (2014) 92(1):49–56. doi:10.1038/icb.2013.54
42. Dirajlal-Fargo S. HIV positive youth who are perinatally infected have impaired endothelial function. *AIDS* (2017) 31(14):1917–24. doi:10.1097/QAD.0000000000001556
43. Amu S, Lavy-Shahaf G, Cagigi A, Hejdeman B, Nozza S, Lopalco L, et al. Frequency and phenotype of B cell subpopulations in young and aged HIV-1 infected patients receiving ART. *Retrovirology* (2014) 11:76. doi:10.1186/s12977-014-0076-x
44. Moody MA, Pedroza-Pacheco I, Vandergrift NA, Chui C, Lloyd KE, Parks R, et al. Immune perturbations in HIV-1-infected individuals who make broadly neutralizing antibodies. *Sci Immunol* (2016) 1(1):aag0851. doi:10.1126/sciimmunol.aag0851
45. Mylvaganam GH, Rios D, Abdelaal HM, Iyer S, Tharp G, Mavigner M, et al. Dynamics of SIV-specific CXCR5+ CD8 T cells during chronic SIV infection. *Proc Natl Acad Sci U S A* (2017) 114(8):1976–81. doi:10.1073/pnas.1621418114
46. Li S, Folkvord JM, Rakasz EG, Abdelaal HM, Wagstaff RK, Kovacs KJ, et al. Simian immunodeficiency virus-producing cells in follicles are partially suppressed by CD8+ cells in vivo. *J Virol* (2016) 90(24):11168–80. doi:10.1128/JVI.01332-16
47. Jiao YM, Yang HG, Huang HH, Tu B, Xing SJ, Mao L, et al. Dichotomous roles of programmed cell death 1 on HIV-specific CXCR5(+) and CXCR5(-) CD8(+) T cells during chronic HIV infection. *Front Immunol* (2017) 8:1786. doi:10.3389/fimmu.2017.01786
48. Xing J, Zhang C, Yang X, Wang S, Wang Z, Li X, et al. CXCR5(+)CD8(+) T cells infiltrate the colorectal tumors and nearby lymph nodes, and are associated with enhanced IgG response in B cells. *Exp Cell Res* (2017) 356(1):57–63. doi:10.1016/j.yexcr.2017.04.014
49. Bai M, Zheng Y, Liu H, Su B, Zhan Y, He H. CXCR5(+) CD8(+) T cells potentially infiltrate pancreatic tumors and present high functionality. *Exp Cell Res* (2017) 361(1):39–45. doi:10.1016/j.yexcr.2017.09.039

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2018 McCarty, Mwamzuka, Marshad, Generoso, Alvarez, Ilmet, Kravietz, Ahmed, Borkowsky, Unutmaz and Khaitan. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



Control of Germinal Center Responses by T-Follicular Regulatory Cells

James B. Wing¹, Murat Tekgüç¹ and Shimon Sakaguchi^{1,2*}

¹ Laboratory of Experimental Immunology, Immunology Frontier Research Center, Osaka University, Suita, Japan,

² Department of Experimental Pathology, Institute for Frontier Medical Sciences, Kyoto University, Kyoto, Japan

Regulatory T-cells (Treg cells), expressing the transcription factor Foxp3, have an essential role in the control of immune homeostasis. In order to control diverse types of immune responses Treg cells must themselves show functional heterogeneity to control different types of immune responses. Recent advances have made it clear that Treg cells are able to mirror the homing capabilities of known T-helper subtypes such as Th1, Th2, Th17, and T-follicular helper cells (Tfh), allowing them to travel to the sites of inflammation and deliver suppression *in situ*. One of the more recent discoveries in this category is the description of T-follicular regulatory (Tfr) cells, a specialized subset of Treg cells that control Tfh and resulting antibody responses. In this review we will discuss recent advances in our understanding of Tfr biology and the role of both Tfr and activated extra-follicular Tregs (eTreg) in the control of humoral immunity.

OPEN ACCESS

Edited by:

Shahram Salek-Ardakani,
Pfizer, United States

Reviewed by:

Luis Graca,
Universidade de Lisboa, Portugal
David H. Canaday,
Case Western Reserve University,
United States

*Correspondence:

Shimon Sakaguchi
shimon@ifrec.osaka-u.ac.jp

Specialty section:

This article was submitted to
T Cell Biology,
a section of the journal
Frontiers in Immunology

Received: 07 June 2018

Accepted: 02 August 2018

Published: 24 August 2018

Citation:

Wing JB, Tekgüç M and Sakaguchi S
(2018) Control of Germinal Center
Responses by T-Follicular Regulatory
Cells. *Front. Immunol.* 9:1910.
doi: 10.3389/fimmu.2018.01910

Keywords: regulatory T-cells (Tregs), T follicular helper (Tfh) cell, T follicular regulatory (Tfr) cell, germinal center (GC), autoimmunity

THE HUMORAL IMMUNE RESPONSE

Antibody driven humoral immunity is essential for host protection from a range of pathogens. This can be broadly separated into the T-independent response, in which B-cell subsets such as B1 cells and marginal zone B cells produce low-affinity antibodies that allow a rapid response to infection, and the T-dependent response in which T-cell help allows the generation of high-affinity antibody and memory immunity over a longer period. Of key importance to T-dependent antibody responses is the germinal center, a structure formed by follicular B-cells and dependent on T-cell help. The germinal center itself is segregated into a dark zone, where centroblast B-cells undergo rapid proliferation and somatic hypermutation (SHM), and the light zone, where higher affinity B-cells are selectively helped by T-cells, allowing them to survive and either be selected as memory or plasma cells, or be recycled back to the dark zone for further rounds of SMH (1). CD4⁺ T-follicular helper (Tfh) cells play a critical role in this process as they are responsible for the majority of T-cell help given to follicular and germinal center B-cells, via delivery of CD40 and IL-21 stimulation to B-cells (2). Tfh form through a multistage differentiation process initiated by contact between dendritic cells (DCs) and pre-Tfh CD4⁺ T-cells. This alone is insufficient to stabilize the full Tfh program, and a second step of prolonged contact between antigen-specific B-cells and the pre-Tfh cell is then required to allow progression to the mature Tfh phenotype. Following this, the Tfh cell can then further differentiate into a highly-activated and germinal center-resident GC-Tfh cell distinguished by high-level expression of CXCR5 and PD-1, in contrast to intermediate levels of both markers expressed by Tfh (2). The chemokine receptor CXCR5 allows

trafficking of the Tfh cell into the B-cell follicle as its ligand CXCL13 is produced by follicular resident dendritic cells and, in humans, by Tfh themselves, allowing further recruitment of new Tfh. Due to their critical role in the generation of high-affinity antibody responses, Tfh cells are vital for the generation of effective humoral immunity. However, dysregulation and unchecked activation of Tfh cells or germinal centers in both humans and mice lead to the production of autoantibodies and lupus-like symptoms, demonstrating the need to tightly regulate the function of these cells (3–5). Additionally, due to their highly mutational nature, germinal centers themselves are a common source of tumorigenesis, meaning that even foreign antigen-reactive germinal center cells require tight regulation (6).

TREGS AND TFR CELLS

Regulatory T-cells (Tregs) expressing the transcription factor Foxp3 are critical for the maintenance of immune homeostasis (7). Signs of a link between certain T-cell populations and the control of humoral immunity have been present since the foundational work that first hinted at the presence of a T-cell population that regulated immunity, demonstrated through the inhibition of anti-sheep red blood cell antibody responses by thymically-derived populations (8). Later, in work leading up to the formal discovery of Tregs, we found that autoantibodies were one of the most sensitive indicators of T-cell autoimmunity (9). When we identified Tregs on the basis of their CD25 expression we found that anti-CD25 depletion of Tregs lead to strong induction of autoantibodies against parietal cells in the stomach epithelia, and against thyroglobulin proteins produced by thyroid follicular cells (10). While CD25 is not entirely exclusive to Tregs, specific depletion of Tregs via diphtheria toxin in mouse models in which Tregs express the primate diphtheria toxin receptor leads to strongly-enhanced GC formation, Tfh cell expansion and antibody responses (11, 12). Loss of control over humoral immunity is also characteristic of mutations of Foxp3 in the scurfy mouse strain and in immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome patients, and leads to the production of autoantibodies, hyper IgE and strongly-enhanced GC/Tfh responses (12–18).

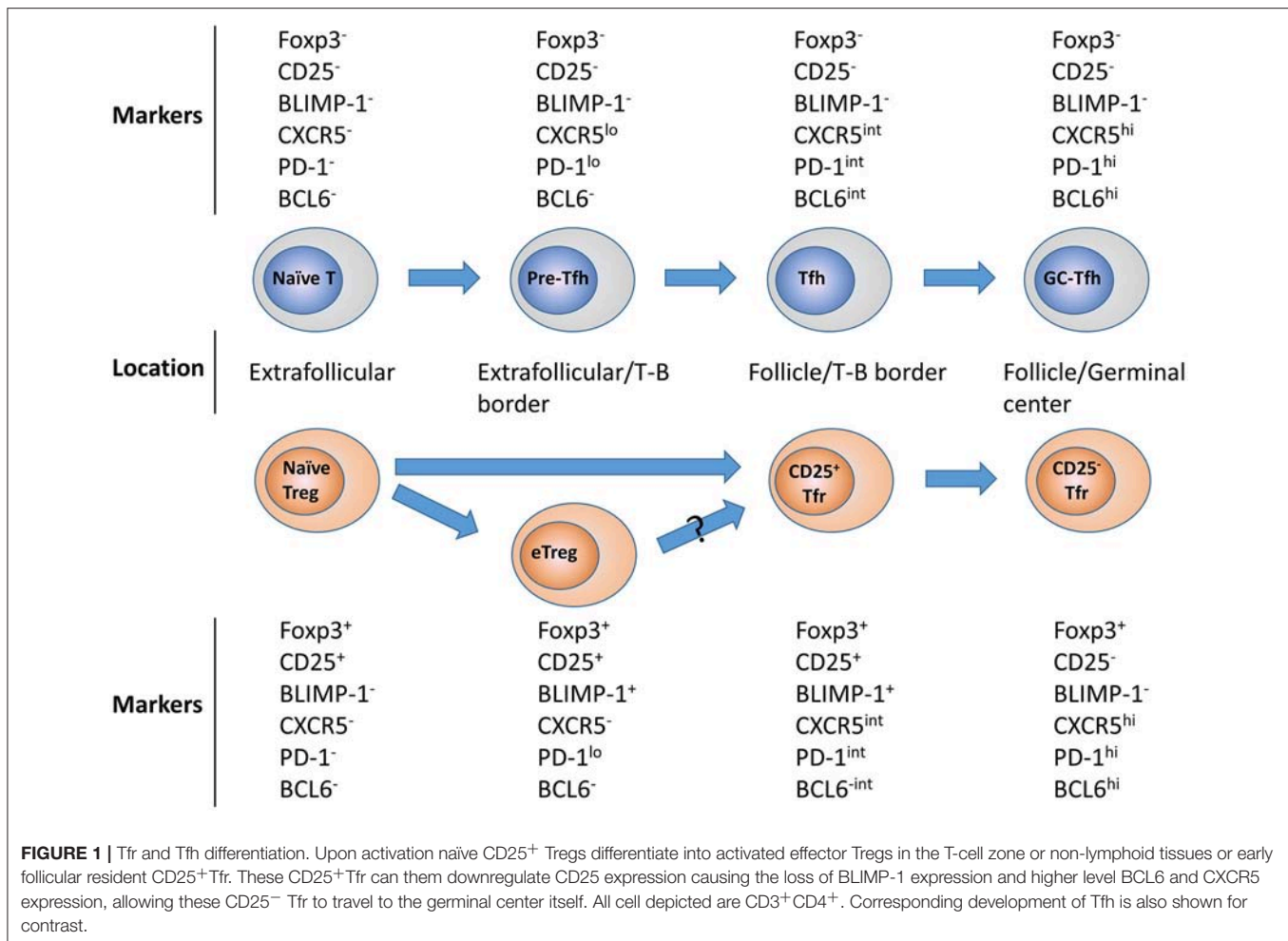
Tregs themselves comprise a number of subpopulations, with some functionally-specialized groups mirroring the transcriptional programming of effector T-cell subsets, allowing them to gain expression of the chemokine receptors responsible for localization to the sites of inflammation in order to suppress the mirrored effector population (19). Early work suggested that, following activation, CD4⁺CD25⁺CD69⁺ Tregs are capable of gaining CXCR5 expression while losing CCR7, a chemokine receptor that homes to the T-cell zone, allowing them to travel to the B-cell follicle to suppress B-cell responses (20, 21). However it was not until 2011 that three groups described Tfr in detail and defined them as CXCR5⁺PD-1⁺BCL6⁺Foxp3⁺ cells (15, 22, 23). While Tfr differentiation is not as well characterized as Tfh, evidence thus far suggests that they have a similar developmental path, with both undergoing a multistage differentiation process

dependent on signals such as CD28, ICOS, SAP and B-cell contact and the Tfh transcription factor BCL6 (15, 22).

Tfr present in the lymphoid organs are an induced subset of effector Tregs. As a result, in a healthy mouse kept in pathogen-free conditions, Tfr are present only in very small numbers in the spleen and lymph nodes, although they can be found in significant numbers in sites of ongoing humoral immune responses, such as the Peyer's patches. In significant contrast to Tregs, Tfr downregulate the IL-2 receptor alpha chain, CD25 (24–26). Downregulation of CD25 appears to be a marker of Tfr development, with CD25⁺ Tfr forming initially, before later formation of more highly-differentiated CD25[−] Tfr. Microscopic analysis of Tfr in the spleens and draining lymph nodes of vaccinated mice reveals that, while the majority of Tfr resident in the follicle and near the T-B border express CD25, almost all germinal center-resident Tfr lack CD25 expression (26). Accordingly, detailed analysis of chemokine receptor and cell adhesion molecules demonstrates that, in keeping with their germinal center localization, CD25[−] Tfr express significantly increased levels of CXCR5, while reducing expression of molecules, such as CCR7 and PSGL-1, responsible for maintenance of localization in the T-cell zones. Further detailed characterization by flow cytometry and RNA-sequencing shows that while CD25⁺ Tfr are more similar to effector Tregs, CD25[−] Tfr have shifted their gene expression signature to a point equidistant between Tfh and effector Tregs, displaying a high level of flexibility in their phenotype. Despite this, they retain stable expression of Foxp3, maintain a characteristic Treg epigenetic signature, and express key Treg suppressive molecules such as CTLA-4, allowing them to suppress both T-cells and B-cells during *in vitro* co-culture (26). Due to their relative similarity to Tfh, it is reasonable to ask if Tfr are formed from thymically-derived Tregs or peripheral Tregs, potentially due to Tfh conversion into Tfr. However, following adoptive cell transfer, both CD25⁺ Tfr and CD25[−] Tfr are formed from naïve CD25⁺Foxp3⁺Tregs, and in agreement with earlier studies showed little evidence that they can form from transferred CD25[−] Foxp3⁺ T-cells (15, 22–24, 26).

While CD25⁺ Tfr in the mouse appear to be at an earlier stage in their differentiation, they are still identifiably Tfr due to their expression of a range of markers at intermediate levels such as CXCR5, PD-1, and BCL6, and localization in the B-cell follicle. As a result of this, we propose a model, in which following initial stimulation, a naïve Tregs bifurcate into eTregs or CD25⁺ Tfr in the follicle, before receiving further activation which allows them to become terminally-differentiated germinal center-resident CD25[−]Tfr. This suggests that in the mouse, CD25⁺ Tfr and CD25[−] Tfr may be the Treg equivalents of Tfh and GC-Tfh, respectively (Figure 1).

A critical question raised by these findings is—why do terminally differentiated Tfr lose CD25 expression? CD25 was the molecule by which Tregs cells were first clearly identified, and is considered both a canonical marker and a critical component for normal Treg function (27). In contrast, IL-2 is known to inhibit Tfh responses, due to STAT5-induced upregulation of BLIMP-1, which inhibits expression of the critical Tfh transcription factor BCL6 (28–30). A further factor to consider is that BLIMP-1 is



expressed by many effector Tregs and plays an important role in their suppressive function by regulating expression of a range of genes such as IL-10 (31, 32). Since Tfr are also a form of effector Treg, this suggests they must maintain a fine balance of these potentially conflicting factors to maintain their phenotype. We and several other groups have demonstrated that addition of IL-2 alongside vaccination or infection in mice inhibits the formation of CD25⁻ Tfr cells while at the same time causing expansion of Tregs (24–26). This is due to a BLIMP-1-dependent mechanism, in which IL-2 causes increased expression of BLIMP-1, which represses expression of BCL6, thus inhibiting Tfr formation (24). As a result CD25⁻ Tfr express only low levels of BLIMP-1 but high BCL6, while CD25⁺ Tfr express higher BLIMP-1 but have only intermediate levels of BCL6 (24, 26). This changing role for IL-2 marks a fundamental split in Treg identity, with the majority of tissue-resident effector Tregs having a BLIMP-1- and IL-2-dependent identity, while fully-differentiated CD25⁻ Tfr depend on BCL6 and are thus inhibited by IL-2. CD25⁻ Tfr can instead be maintained by the presence of other cytokines and signals such as IL-4, which is highly produced by Tfh (2, 26). It is also the case that CD25⁻ CXCR5⁻ BCL6⁻ Foxp3⁺ Tregs at tissue sites of

inflammation can be maintained in an IL-2 independent manner (33).

While it is clear that a large proportion of Tfr downregulate CD25 in mice, recent results examining human Tfr suggest that downregulation of CD25 may be less characteristic of human Tfr. Sayin et al. demonstrate via microscopy that the majority of Tfr detectable in the follicles of human mesenteric lymph nodes express CD25, and that the cells are highly concentrated at the T-B border but not the GC itself (34). Interestingly, while microscopy suggested that essentially all the Tfr in the B-cell follicle and GC itself were CD25⁺, flow cytometry analysis in the same report demonstrates that PD-1^{hi} Tfr express significantly less CD25 than PD-1^{int} or negative Tfr (CD25 MFI 616 ± 96 vs. 1101 ± 121.4, $p = 0.0074$ unpaired t -test), and also display a bimodal expression of CD25 with a significant fraction appearing to be CD25^{lo/-} (34). This is in keeping with two previous reports that suggested that the most highly-differentiated PD-1^{hi} CXCR5^{hi} BCL6⁺ Tfr in human tonsils also downregulate CD25 (25, 26). Importantly, however, while PD-1^{hi} Tfr do appear to be enriched in the GC itself, they are extremely rare, with only around 3% of Tregs in the mLN matching this description (34).

Similarly we found around 5% in the tonsils (26). As a result the ratio of Tfh/Tfr is skewed heavily to Tfh in the GC region, which may indicate that Tfr outside the GC itself are most critical in humans (34) and may also explain why human lymph node resident-Tfr are resistant to rituximab induced depletion of GC B-cells (35). In contrast, the mouse appears to have a greater number of Tfr in the GC itself, and a correspondingly larger fraction of CD25⁺ Tfr.

IL-21, a characteristic Tfh cytokine, may play a role in the maintenance and differentiation of Tfr. IL-21 has been demonstrated to indirectly affect Tregs homeostasis by suppressing IL-2 production by Tconv cells (36). However, more recently, cell-intrinsic roles for IL-21 on the formation of Tfr have been described (37, 38). Autoimmune-prone BXD2 mice lacking IL-21 production have their Tfh/Tfr ratio skewed toward Tfh. This appears to be due to both direct effects on Tfh STAT3 signaling, and possibly indirectly via Akt signaling in Tfr (37). Jandl and colleagues found that Tregs lacking IL-21R have an increased proportion of Tfr among total Tregs. Further, the proportion of Tfr that express CD25 was increased by a reduction in IL-21, which would otherwise induce BCL6-mediated downregulation of CD25 expression. When IL-21R-deficient or WT Tregs were transferred into Treg-depleted mice, followed by vaccination, loss of IL-21R expression by Tregs was associated with reduced antigen-specific antibody production. Interestingly, this loss of antigen-specific antibody was marked by a reduction in the percentage of antigen-specific B-cells within the GC but no change in the total number of GC-B-cells. While it was not examined in this case, this would imply that there was a proportional gain in non-antigen-specific or autoreactive B-cells in the same system (38). As a result it seems that IL-21 can prevent BCL6-driven downregulation of CD25, and thus enhance IL-2-driven Tfr proliferation. However, as noted earlier, IL-2 itself inhibits Tfr differentiation via BLIMP-1-dependent inhibition of BCL6 (24–26). These results may seem contradictory, however a key point here is to indicate the split between CD25⁺ and CD25⁺ Tfr. We found that supplementation with IL-2 results in an almost total loss of CD25⁺ Tfr but CD25⁺ Tfr are retained. Equally, CD25⁺ Tfr are preferentially expanded in IL-21R-deficient Tregs. This suggests that IL-21 and IL-2 may control the balance between CD25⁺ and CD25⁺ Tfr, since IL-2 enhances the proliferation of naïve and eTregs (which are the precursors of Tfr) and CD25⁺ Tfr in the follicle, while blocking full differentiation into germinal center-resident CD25⁺ Tfr. On the other hand, IL-21 may encourage CD25⁺ Tfr to fully differentiate into CD25⁺ Tfr via its effects on BCL6-mediated downregulation of CD25, but this comes at the price of reduced IL-2-dependent proliferation by CD25⁺ Tfr.

THE *IN VIVO* ROLE OF TFR AND CONTRIBUTION OF TREGS TO HUMORAL IMMUNITY

Studies into the exact *in vivo* role of Tfr have yielded conflicting results. Several initial studies used adoptive transfer systems to study the function of Tfr. Here, they transferred CXCR5- or

BCL6-deficient Tregs into T-cell-deficient mice, alongside WT CD4⁺ Foxp3⁺ cells, before vaccinating them. Loss of Tfr function in this system caused an increase in the number of germinal center B-cells while also increasing the amount of antigen-specific antibody, albeit with reduced affinity (15, 23). Another study used bone marrow chimeras of SAP-deficient and Foxp3-deficient bone marrow. These mice lack Tfr, since the Foxp3-sufficient cells lack SAP, which is critical for Tfr development. In this system, GC and Tfh numbers were increased but antigen-specific antibody production was reduced, presumably due to increased expansion of self-reactive Tfh (22). Treg-specific inhibition of TRAF3-dependent ICOS expression and a resulting defect in Tfr formation caused an increase in the number of GC B-cells, with no change in Tfh cell number but rather increased cytokine production by these cells which in turn also resulted in increased SHM (39). Reduced Tfr infiltration into the follicles due to loss of NFAT2-dependent CXCR5 expression also resulted in increased GC cell numbers and antigen-specific antibody production (40). The transcription factor Helios is also expressed by the majority of Tregs and Tfr. Mice with a Treg-specific loss of Helios expression develop autoimmunity characterized primarily by enhanced autoantibodies, GC size and Tfh cell number. This appears to be primarily due to loss of Tfr cell function, although some other abnormalities, such as an unstable phenotype and gain of pro-inflammatory cytokine production by Tregs, suggests that this phenotype may also be partly attributable to a wider loss of Treg function (41). Further to this, recent work demonstrates an essential role for mTOR complex 1 (mTORC1) signaling that induces STAT3-TCF1-driven induction of BCL6 expression (42). As a result, when essential components of the mTORC1 pathway were genetically depleted, Tfr development and function were impaired, leading to enhanced numbers of GC B-cells and Tfh following vaccination.

Altogether, these results suggest that Tfr control GC cell number and Tfh function but have varied effects on the quality and antigen specificity of the response. Conditional knockout of BCL6 in Tregs via the *cre-lox* (BCL6-flox: Foxp3-cre) system promises the ability to analyse Tfr function in more detail than is possible with cell transfer or bone marrow chimera approaches, and without some of the caveats that come with loss of function in genes that may also affect broader Treg functionality. Using this system, the Dent group found that Tfr were, as expected, significantly reduced, but this had no effect on either GC B-cell or Tfh cell numbers. However, Tfh production of IFN- γ , IL-10, and IL-21 were increased, resulting in increased IgA production but a reduction of IgG in the context of vaccination with sheep red blood cells or NP-KLH. In contrast, in pristine induced lupus models, dsDNA autoantibody specific IgA was increased in the absence of a clear effect on IgG. However, when using the same mouse model with a DNA prime-protein boost vaccination, the IgG titer was not affected but antibody avidity was reduced, suggesting a role for Tfr in the control of the quality of the antibody reaction (43). In contrast, another group demonstrated that Tfr are critical to IgA selection in the gut and that their presence increases the production of IgA-positive plasma cells in the lamina propria. This in turn has a critical role in the regulation of the microbiota via IgA (44).

Further work in an influenza virus infection system demonstrates that, in this situation, Treg-specific BCL6 deficiency induced no change in GC and Tfh cell numbers, but caused a clear increase in the number of antibody-secreting plasma cells. However, this also coincided with a reduction in the proportion of antigen-specific cells, while increased autoantibody production was also observed when the infected mice were treated with IL-2 in order to suppress Tfr function (24). These findings were recently built on, with another group using BCL6-flox Foxp3-cre mice to demonstrate that—again—loss of Tfr had no clear effect on GC or Tfh cell number in influenza infection, but that influenza specific IgG2c antibody production was slightly, but significantly, increased (45). While these mice were healthy at a young age, by 30 weeks they had developed immune infiltration of several organs such as the lung, pancreas, and salivary gland, while also developing autoantibodies. In contrast to influenza infection, increased numbers of GC and Tfh were seen, suggesting that Tfr may control self-reactive GCs to a larger extent than non-self-reactive responses (45).

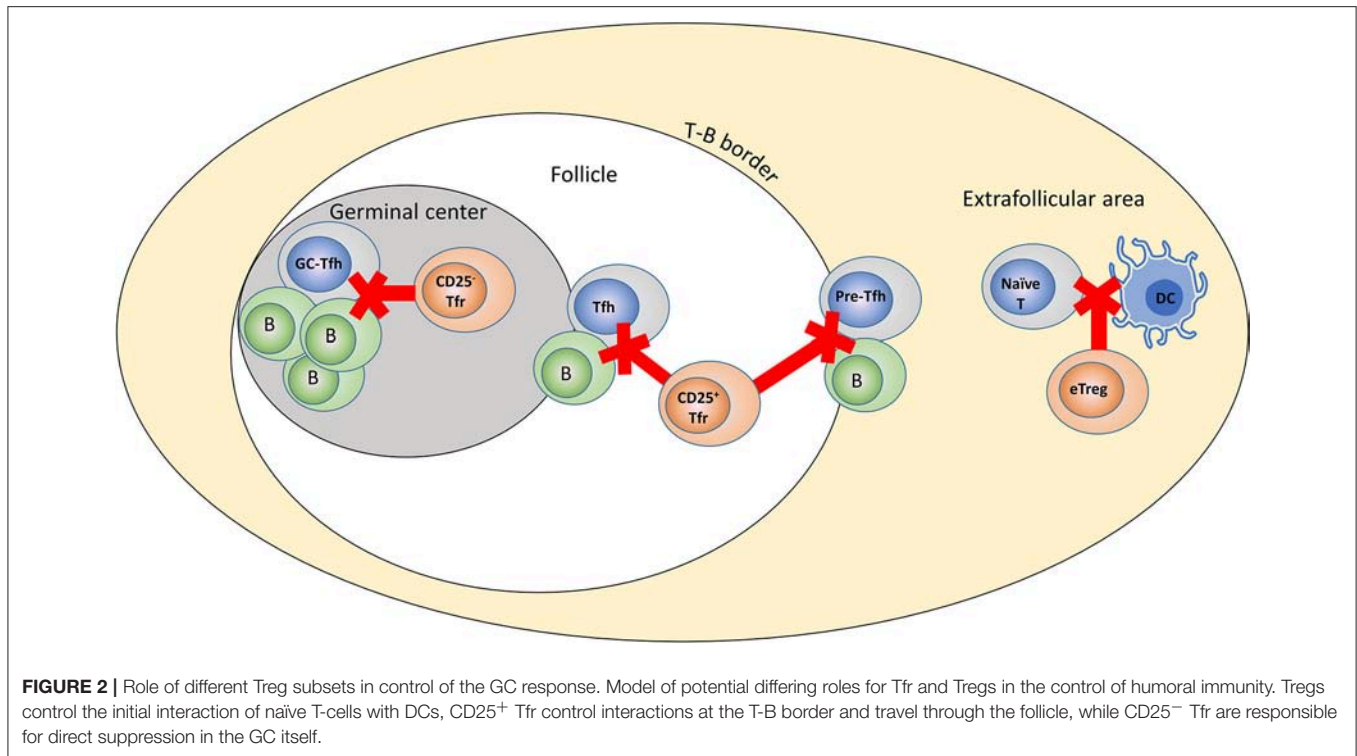
While it is clear that Tfr play an important role in the control of antibody production, whether they primarily control GC B-cell numbers or have a subtler role in the control of the quality and specificity of the antibody response remains less clear. Recent studies using conditional genetic deletion of BCL6 in Tregs do not suggest a role for Tfr in the control of overall GC numbers during the response to foreign antigens, but instead see a more subtle role in modulating antibody production via plasma cells. In contrast, in the context of a primarily self-antigen-driven response, Tfr may play a more active role directly controlling GC B-cell and Tfh cell numbers. This may be because Tfr have a more self-skewed TCR repertoire, respond better to vaccination with self-antigens rather than foreign antigens, and do not appear to require recognition of the same antigen as a particular Tfh cell in order to suppress it (25, 46, 47). We also previously demonstrated that while short-term depletion of Tregs/Tfr is effective at enhancing antigen-specific Tfh formation following vaccination with a foreign antigen, longer-term depletion of Tregs further increased the total number of Tfh but also reduced the absolute number of antigen-specific Tfh (12). This indicates that while partial or temporary disruption of Treg/Tfr function leads to increased availability of co-stimulatory molecules, and a resulting increase in the antigen-specific immune response, prolonged or total disruption of Treg/Tfr function may lead to a more profound loss of immune homeostasis resulting in aggressive expansion of autoreactive cells, which outcompete antigen-specific cells and cause skewing to self-reactive responses. As a result it is possible that a more or less complete loss of function in Tfr may have differing effects.

The relatively subtle effects of specific Tfr depletion stand in considerable contrast to the large effects seen when Tregs as a whole are depleted (11, 12). Equally, while the most highly-differentiated Tfr lack CD25 expression, anti-CD25 antibody is capable of inducing substantial autoantibody production (10). Given that it takes several days for Tfr to form following initial stimulation it is reasonable to surmise that Tregs outside the follicle are responsible for the control of the initiation stages of Tfh formation, while Tfr may be more critical a later in the

process. We suggest a model in which CXCR5⁺ Tregs, CD25⁺ Tfr present in the follicle and T-B border, and CD25⁺ Tfr present in the GC have distinct roles at different points following initial stimulation of a GC reaction, essentially forming three rings of protection for the prevention of autoreactive GCs (**Figure 2**). Specifically, CXCR5⁺ Tregs may control the initial formation of Tfh via suppression of the contact between DCs and naïve T-cells, CD25⁺ Tfr in the follicle may interfere with contact and signaling between Tfh and B-cells at the T-B border or during transit through the follicle, while CD25⁺ Tfr resident in the GC itself may interfere with the interactions between GC-Tfh and centrocytes. This division is likely to be temporal as well as spatial, with the key events in the extra-follicular region occurring earlier than events in the GC. Even in the context of an established GC reaction, the outer rings of defense may still be critical to prevent new autoreactive cells infiltrating an existing GC as given sufficient antigen naïve B-cells and newly formed Tfh are capable of entering pre-existing GCs even at relatively late stages of their life cycle (48, 49). The lines between these rings of defense are likely to be blurred for several reasons: Tregs, CD25⁺ Tfr and CD25⁺ Tfr are developmentally related and a single cell could potentially perform in all three areas over the course of its differentiation, also, almost all Tfr in the GC lack CD25 expression the follicle contains a mixture of CD25⁺ and CD25⁺ Tfr which may represent GC Tfr cells traveling back to the follicle in a manner similar to Tfh (48). With these caveats in mind, we believe this model may capture the essence of the division of labor between these Treg subsets. While loss of CD25 appears to be a good marker of Tfr differentiation in mice, in humans CD25⁺ Tfr located in the GC seem to be rare, so it may be the case that human Tfr are weighted to a greater role for CD25⁺ Tfr in the B-cell follicle and T-B border (34). As a result this model may be a good fit for the murine system while further detailed experiments are required to better understand human Tfr biology.

MECHANISMS OF TFR FUNCTION

A number of suppressive mechanisms have been proposed to have a role in Tfr function. CTLA-4 is known to be critical to Treg suppressive function to the extent that its specific deletion in Tregs leads to severe autoimmunity similar to that seen in Foxp3-deficient scurfy mice (50). We and others previously demonstrated that loss of CTLA-4 function in Tregs had a severe impact on the suppression of Tfh responses (12, 51, 52). CTLA-4 primarily acts to deplete the CD28 ligands CD80 and CD86 from the surface of antigen-presenting cells, preventing them from providing co-stimulation to T-cells (53). It is likely that this mechanism is of primary significance in controlling the initial stages of Tfh cell formation at the T-B border, since blockade of CD80 and CD86 has little effect on already preformed Tfh cells, while mice lacking CD80 and CD86 on B-cells but not DCs were still able to form Tfh and GCs, suggesting that the core function cell extrinsic function of CTLA-4 may be at the early stage of GC formation during contact with DCs (54, 55). However, several groups have also found that expression of CD80



and CD86 by B-cells alone are sufficient to induce germinal center reactions in mice otherwise lacking CD80 and CD86 (12, 56). In both cases, a cell-intrinsic role for B-cell CD80 and/or CD86 was described, since when transferred together CD80/86 sufficient B-cells were better able to form GC B-cells than CD80/86 deficient B-cells (12, 56). Further to this, a B-cell-intrinsic role for CD80 in controlling Tfh and plasma cell formation was also observed (57). Together these results would suggest that in specific circumstances, CD80 and CD86 expression on either DCs or B-cells may be dispensable but that it is likely that optimal Tfh responses require both. Aside from its ligand-depleting function, CTLA-4 has also been suggested to mediate direct suppression of B-cell antibody production by putative Tfr (CD25⁺CD69⁻), although the molecular events underpinning this remain unclear (21). It is also possible that cell-intrinsic functions of CTLA-4 that act on the Tfr itself may have a role in the control of later-stage GC reactions (51) as at least some of the effect of CTLA-4 appears to be independent of CD28 signaling (58).

Another recently proposed mechanism of Tfr function is expression of the IL-1 decoy receptor IL-1R2 (25). Addition of IL-1 to vaccinated mice enhances the Tfh response and resulting antibody production, while blocking IL-1 has the opposite effect. Tfr have enhanced IL-1R2 expression in comparison to other Tregs and are able to inhibit IL-1 driven enhancement of Tfh cytokine production in a similar manner to blocking IL-1 (25). This suggests that Tfr may be able to engage IL-1 and prevent its interaction with Tfh cells. RNA sequencing reveals that IL-1R2 is expressed by both CD25⁺ and CD25⁻ Tfr (26). Further work is required to determine the *in vivo* importance of this proposed

mechanism. Additionally, IL-1R2 is also highly expressed by both tumor-infiltrating and CXCR3⁺T-bet⁺ pancreatic Tregs, suggesting that it may be a mechanism used by a range of highly-activated Tregs subtypes, including Tfr (59, 60).

GARP, a Treg surface molecule that supports the anchoring of latent TGFβ onto the surface of Tregs, has also recently been shown to be enriched on the surface of human Tfr, although again further work is required to determine its exact *in vivo* contribution to Tfr function (34). TGFβ signaling has been shown to control Tfh numbers, although the phenotype is much more severe than loss of Tfr function alone, suggesting that it may be involved at multiple stages of Tfr/Treg function (61).

Tregs have also been suggested to directly kill activated B-cells via production of granzymes and perforin (62, 63). This work was carried out before the full description of Tfr, but it seems likely that these cells were primarily naïve/eTregs since they were purified on the basis of CD25 expression from healthy wild-type mice.

Surprisingly, recent work suggests that Tfr may also have some positive role in the modulation of the GC response. Some earlier results suggest that Tfr may support specific IgG production and affinity maturation in at least some contexts (22, 43). Building on this, recent results demonstrate that production of the cytokine IL-10 by Tfr enhances germinal center responses by driving germinal center B-cells into a proliferative dark zone phenotype via induction of the transcription factor FOXO1. As a result, specific knockout of IL-10 in Tfr results in reduced GC cell numbers (64). In contrast, IL-10 receptor-deficient T-cells more readily develop into Tfh cells, suggesting that IL-10 signaling into B and T-cells may have differing effects on GC and Tfh formation

(65). The finding that Tfr have at least some ability to support the germinal center reaction differs from the results from models that look at total loss or inhibition of Tfr function, and suggests that while their overall contribution can probably be characterized as suppressive, Tfr may have a complex role in the fine-tuning of the germinal center response that includes the delivery of some conflicting signals (66).

In short, a number of potential mechanisms of Treg/Tfr control of humoral immunity have been identified. Further analysis of RNA sequencing data obtained from Tregs, CD25⁺ Tfr and CD25⁻ Tfr revealed that these cells have differing expression of various Treg suppressive molecules (26) (**Figure 3**). Importantly we use activated (CD44⁺CD62L⁻CXCR5⁻) but not Naïve Tregs as a baseline comparison to Tfr. Since Tfr themselves are all CD44⁺CD62L⁻ their direct comparison to total CXCR5⁻ Tregs which contain a significant proportion of naïve cells will lead to the identification of a range of effector Treg markers being misidentified as Tfr enriched. Granzyme B appears exclusive to eTregs, IL-10 is present in CD25⁺ Tfr and eTregs, IL1-R2 is concentrated in the CD25⁺ Tfr, CD73 is slightly increased in both Tfr subsets, CD39 and CTLA-4 appear to favor eTregs. However only IL-10 and granzyme B were identified as differentially expressed between these Treg groups, while CTLA-4 protein was also confirmed to not be significantly different by flow cytometry (26). In all cases Tfh themselves express lower levels of these molecules. As a result, it seems Tfr may act via a number of suppressive mechanisms and that all of these functional molecules are shared with Tregs.

While the mechanisms of Tfr function are still under investigation, it is of interest that Tfr induce sustained suppression of Tfh and B-cells that outlasts the immediate contact between these cells. B-cells that have been previously suppressed by Tfr have altered metabolic and epigenetic programming that impair their ability to respond to later stimulation by Tfh cells in the absence of Tfr cells (67). This suggests that the effect of Tfr suppression persists beyond the actual contact period between the Tfr and B-cell/Tfh, and may be critical in the generation of a large effect from a small number of cells, since Tfr are significantly outnumbered by B-cells and Tfh in the follicular environment, particularly in human GCs (34).

CIRCULATING TFR

While bona-fide Tfr are defined by their localization in the B-cell zones of lymphoid organs, CXCR5⁺Foxp3⁺ circulating Tfr (cTfr) can also be found in blood. Due to the relative difficulty in obtaining samples of human lymphoid tissues, this population has been the focus of investigations in humans.

cTfr in mice appear to be formed early in the Tfr differentiation process since they are retained even in μ MT B-cell deficient mice, suggesting that they are dependent on the initial contact with DCs but do not require the second contact with B-cells that would normally finalize the differentiation process (46, 68). Similarly in humans cTfr cells were generated in the peripheral lymphoid tissue following the initial activation mediated by DCs, but were similarly not affected by a lack of B-cells demonstrating

an early bifurcation of cTfr and tissue-resident Tfr (68). As a result there are significant phenotypic differences between cTfr and from Tfr isolated from tonsils and other lymphoid organs with cTfr having much reduced expression of normally characteristic markers such as PD-1, ICOS and BCL6 (26, 46, 68, 69). This difference is further emphasized by the finding that Tfr from human tonsils have a subset of CD25^{negative/lo} BCL6⁺Foxp3⁺ Tfr while CD45RA⁻ effector Tfr in blood are CD25^{int}. In contrast CXCR5⁻ effector Tregs in blood upregulate CD25 in comparison to naïve Tregs, again demonstrating the significant difference in IL-2 metabolism between Tfr and other effector Tregs (26). Interestingly due to their CD25^{int} nature CD45RA⁻ cTfr find themselves in the fraction (FRIII) of blood Tregs that we previously identified as primarily Foxp3^{int}CD25^{int} non-Tregs (70). However, since we and others have confirmed that cTfr retain stable expression of Foxp3, a Treg-type demethylation signature, and suppressive function, it seems that FRIII may contain a mixture of Tregs and non-Tregs (26, 68, 71). This mixed nature of FRIII was recently confirmed by the finding that CD127⁻CD25^{int}CD45RA⁻ FRIII Tregs can be further divided into CD49d⁺CCR4⁻, CCR4⁻CD49d⁻, and CCR4⁺CD49d⁻ cells, with the CD49d fraction expressing inflammatory cytokines (72). In our hands CD45RA⁻CD127⁻CXCR5⁺CD25^{int} cTfr lack CCR4 and CD49d expression and, as a result, cTfr make up the majority of CCR4⁻CD49d⁻ cells in FRIII (26). This suggests that FRIII can be stratified into CD49d⁺CXCR5⁻CCR4⁻ non-Tregs, CD49d⁻CXCR5⁺CCR4⁻ cTfr and CD49d⁻CXCR5⁻CCR4⁺ Tregs.

Surprisingly, given that CXCR5 is normally considered an activation/memory marker in T-cells, some Tfr found in peripheral blood appear to have a naïve phenotype (CD45RA⁺CXCR5⁺) (68, 69, 73). Similar to Tfr themselves, the first indication of the presence of these cells can be found in the work of Lim et al. in 2006 who found that, in contrast to a range of other chemokine receptors normally associated with effector/memory cells, such as CCR2, CCR4, and CCR6, CXCR5 expression by Tregs was increased on CD45RA⁺ cells in a similar manner to CCR7 (73). Expression of markers such as CD45RA would normally be considered an indication of naïve status. However, these cells are absent in cord blood and the thymus, suggesting that they are induced from truly naïve Tregs by stimuli that occur after birth (68, 73). Given that CD45RA⁺ Tregs are now considered a promising target for *in vitro* expansion and clinical use, this phenomenon may require further investigation (74).

While it is clear that cTfr retain the ability to suppress T-cells *in vitro*, there are conflicting reports of their ability to block B-cell antibody production. One group found that these cells were unable to suppress antibody production (68) while several groups found the opposite (26, 35, 71). Interestingly, Liu et al., found that cTfr suppressive activity increased in correlation with the sequential shift of cTfr from FRIII Foxp3^{int} into a highly-activated Foxp3^{hi} phenotype from healthy donors > active RA patients > patients in remission.

One unifying feature of these studies is that CXCR5⁻ Tregs in circulation are capable of suppressing antibody responses *in vitro* at least as well as, and sometimes better than, cTfr (26, 68). This

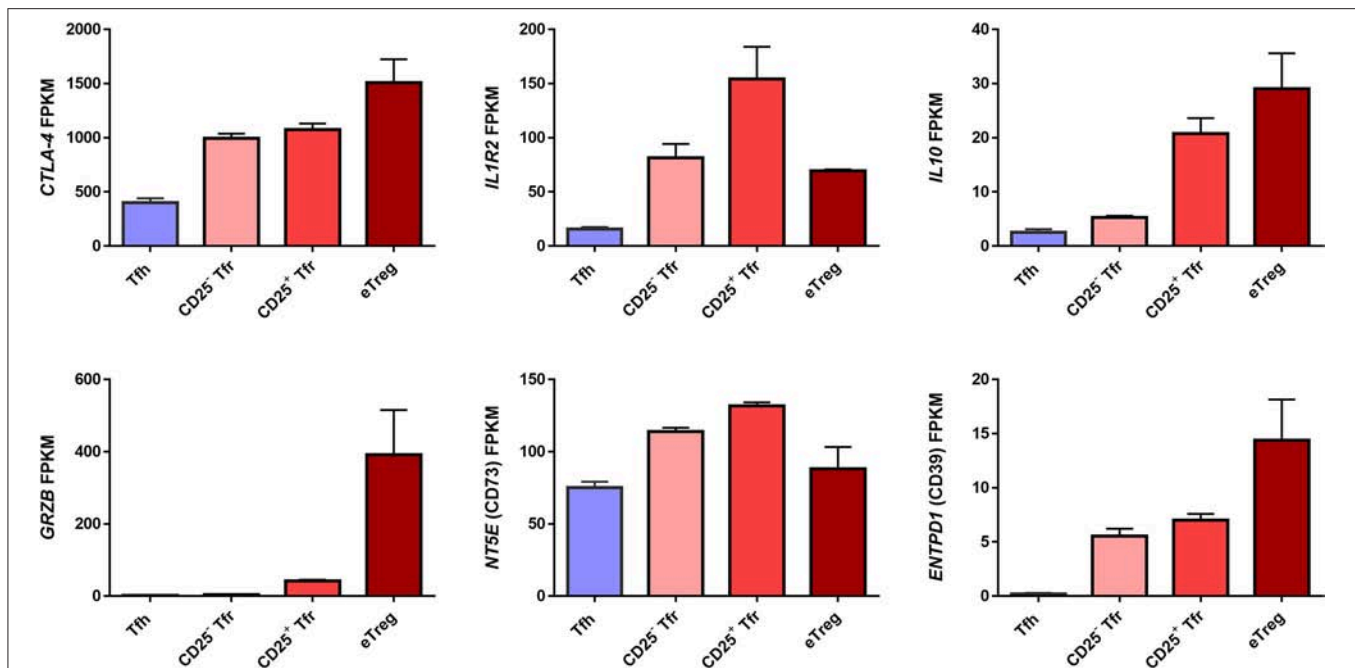


FIGURE 3 | Expression of suppressive genes by Tfh, Tregs, CD25⁺ Tfr, and CD25⁻ Tfr. RNA expression of murine Tfr cells measured as Fragments Per Kilobase Million (FPKM) taken from RNAseq dataset in Wing et al. (26). $n = 2 \pm \text{SEM}$. Mice were vaccinated with NP-Ova in alum and cells sorted from peripheral lymph nodes 7 days later. CD4⁺B220⁻ cells from Foxp3 reporter were sorted as CD62L⁻CXCR5⁺PD1⁺Foxp3⁻GITR⁻ Tfh, CD62L⁻CXCR5⁻Foxp3⁺GITR⁺CD25⁺ eTreg, CD62L⁻CXCR5⁺PD1⁺GITR⁺CD25⁺ CD25⁺ Tfr, and CD62L⁻CXCR5⁺PD1⁺Foxp3⁺GITR⁺CD25⁻ CD25⁻ Tfr.

might be considered an indication that these cells may not be bona fide Tfr. However, whether the ability to suppress humoral responses *in vitro* should be considered a defining property of Tfr is questionable. CXCR5⁻ Treg from the tissues of both humans and mice are fully capable of suppressing B-cell antibody production *in vitro* (12, 26, 34). While certain suppressive mechanisms may skew toward Tfr or eTregs (Figure 3), and may have different roles at different points of the humoral response, it seems likely that none of them are entirely exclusive. This is emphasized by the finding that CTLA-4, IL-10, and IL-1R2 all have roles in both Tfr and Treg suppressive function. This current lack of evidence for a suppressive mechanism which is unique to Tfr, and which might explain any specific ability to suppress humoral immunity by Tfr, suggests that the capacity to suppress B-cells *in vitro* may not be a defining characteristic of Tfr. Instead we favor a model in which the key *in vivo* difference between Tregs and Tfr is not their mechanism of suppression, but rather their localization. Simply, we suggest that both CD25⁻ and CD25⁺ Tfr are able to act at different points of the humoral response from Tregs, because they are in the right place to do so, a distinction that is lost during an *in vitro* assay.

TFR IN HUMAN DISEASE

Due to their relatively recent discovery, the role of Tfr in human disease is not well understood at this time. The proportion of cTfr in human blood may be a direct indicator of the extent of ongoing antibody responses. The total number of cTfr in

blood increases after vaccination, while the proportion of them that are CD45RA⁺ drops (68, 69). cTfr are also increased as a proportion of Tregs in patients with ongoing Sjögren's syndrome (SS), and the Tfr/Tfh ratio strongly correlates with both autoantibody production and activated (PD-1⁺ICOS⁺) T-cell infiltration into the minor salivary glands of patients (68, 75). In the context of infection, cTfr expand during chronic viral and parasitic infections such as human immunodeficiency virus (HIV), hepatitis B virus (HBV), hepatitis C virus (HCV), and *Schistosoma japonica* (76–78). The increase of cTfr cell frequency in patients chronically infected with either HBV or HCV showed strong correlation with serum viral load in both infections. In rheumatoid arthritis, increased percentages of cTfr, decreased percentages of Tfh and a corresponding drop in the ratio of Tfh/Tfr was associated with stable disease and reduced levels of autoantibodies, while active disease was correlated to increased cTfr but no change in the Tfh/Tfr ratio (71). However, treatment may cause cTfr numbers to drop resulting in a high Tfh/Tfr ratio but no clear relationship between cTfr numbers and autoantibodies (79). Similarly to untreated RA patients, the Tfh/Tfr ratio was correlated with autoantibody production in SLE patients, although in this case this was due to a loss of Tfr while the proportion of Tfh remained stable (80). cTfr cell frequency was also reduced in the blood of multiple sclerosis patients and these cells were found to be less suppressive compared to those of healthy controls (69). Together these results suggest that an increased proportion of cTfr in the blood is a marker of ongoing humoral activity and that the Tfh/Tfr ratio may give an indication

of autoantibody production. However in the cases of SLE and MS this correlation seems less clear. Whether, as seems possible, this is an indication that autoantibody production in these settings is due to a proliferative defect in cTfr is unclear at this time.

The frequency of cTfr cells in primary immunodeficiency disorders also displays variability. Cunill et al. reported that the smB⁺ (switched memory phenotype B-cell deficient) subset of common variable immunodeficiency (CVID) patients showed remarkable reduction in their blood CXCR5⁺CD25^{hi}CD127^{low} Tfr cell numbers (81). Store-operated Ca²⁺ entry (SOCE) via Ca²⁺ release-activated Ca²⁺ (CRAC) channels mediated by STIM and ORAI proteins is an essential signaling pathway in T cells, and it controls both Tfh and Tfr cell differentiation (82). Vaeth et al. demonstrated that frequency of CD45RO⁺Foxp3⁺Helios⁺Tfr-like effector Treg cells is significantly diminished in patients with severe combined immunodeficiency-like disease, characterized by inherited loss-of-function mutations in STIM1 or ORAI1 genes (82). Meanwhile, Jandl et al. found that the percentages of CD4⁺CXCR5^{hi}PD-1^{hi}CD127^{low} Foxp3⁺ cTfr cells were elevated in the peripheral blood of IL-21R-deficient patients compared to healthy controls (38). Despite this the total frequency of cTfr seem surprisingly resistant to a range of mutations that affect Tfh formation, such as STAT1 and STAT3, IL21R, IL10R, and ICOS (83).

TFR IN TUMORS

Increased numbers of activated ICOS⁺CXCR5⁺ Tfr are seen in the blood of non-small cell lung cancer patients although this did not correlated with disease stage (84). Tfr are also enriched in the lymph nodes of diffuse large B cell lymphoma (DLBCL) patients and the proportion of Tfr was reduced in patients with more advanced stage disease (85). It is unclear what the role of Tfr may be in the tumor environment but Tfh like cell infiltration has been demonstrated to be predictive of survival in breast cancer and may drive ectopic germinal centers (86). Although in this case these were CXCR5⁺PD-1^{hi}CXCL13 T-helper identified in inflamed joints and breast cancer (described as TfhX13) (87). TfhX13 and Tfh appear closely related, sharing most of the transcriptional programming related to B-cell help but with differing homing capabilities as Tph primarily infiltrate inflamed tissue in a CXCR5 independent manner. It is unclear if

Tregs have a direct equivalent for these cells but in breast cancer PD-1^{int}ICOS^{hi}CXCR5⁺ Tregs are seen infiltrating the same areas suggesting that these may be non-Tfr effector Tregs (87).

CONCLUSION

Due to their recent discovery the Tfr field is still young and many important questions about the formation and function of Tfr and their role in a range of antibody driven autoimmune diseases remain unanswered. Recent work examining specific knockout of Tfr suggests that Tfr may be biased to the control of autoantibody responses while having a more subtle role on the production of non-self-antibodies. It seems clear that in mice Tfr readily lose expression of CD25 and this correlates with a germinal center localization a CXCR5^{hi}BCL6^{hi}PD-1^{hi} phenotype. In humans these cells appear to be less common suggesting that CD25⁺Tfr may have a more dominant role in this setting. However the relative contributions and exact suppressive mechanisms used by CD25⁺Tfr, CD25⁺Tfr and Tregs at different points in the regulation of the humoral immune response remain unclear and may prove hard to separate due to their highly interrelated nature. New tools may be needed to separate these populations, particularly in humans in which our understanding of Tfr biology is limited, making further human Tfr studies an ongoing priority.

AUTHOR CONTRIBUTIONS

JW conceived and wrote the manuscript and prepared figures. MT and SS contributed to the writing and revision of the manuscript.

FUNDING

JW was supported by Japan Society for Promotion of Science (JSPS) grant in aid for specially promoted research 16H06295 and C grant 18K07175; SS was supported by JSPS grant in aid for specially promoted research 16H06295.

ACKNOWLEDGMENTS

The authors thank Dr. Ee Lyn Lim for critical reading of the manuscript.

REFERENCES

- Klein U, Dalla-Favera R. Germinal centres: role in B-cell physiology and malignancy. *Nat Rev Immunol.* (2008) 8:22–33. doi: 10.1038/nri2217
- Crotty S. T follicular helper cell differentiation, function, and roles in disease. *Immunity* (2014) 41:529–42. doi: 10.1016/j.immuni.2014.10.004
- Linterman MA, Rigby RJ, Wong RK, Yu D, Brink R, Cannons JL, et al. Follicular helper T cells are required for systemic autoimmunity. *J Exp Med.* (2009) 206:561–76. doi: 10.1084/jem.20081886
- Vinuesa CG, Sanz I, Cook MC. Dysregulation of germinal centres in autoimmune disease. *Nat Rev Immunol.* (2009) 9:845–57. doi: 10.1038/nri2637
- Simpson N, Gatenby PA, Wilson A, Malik S, Fulcher DA, Tangye SG, et al. Expansion of circulating T cells resembling follicular helper T cells is a fixed phenotype that identifies a subset of severe systemic lupus erythematosus. *Arthritis Rheum.* (2010) 62:234–44. doi: 10.1002/art.25032
- Robbiani DF, Bunting S, Feldhahn N, Bothmer A, Camps J, Deroubaix S, et al. AID produces DNA double-strand breaks in non-Ig genes and mature B cell lymphomas with reciprocal chromosome translocations. *Mol Cell* (2009) 36:631–41. doi: 10.1016/j.molcel.2009.11.007
- Sakaguchi S, Yamaguchi T, Nomura T, Ono M. Regulatory T cells and immune tolerance. *Cell* (2008) 133:775–87. doi: 10.1016/j.cell.2008.05.009
- Gershon RK, Kondo K. Cell interactions in induction of tolerance - role of thymic lymphocytes. *Immunology* (1970) 18:723–37.

9. Sakaguchi S, Wing K, Miyara M. Regulatory T cells - a brief history and perspective. *Eur J Immunol.* (2007) 37:5116–23. doi: 10.1002/eji.200737593
10. Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J Immunol.* (1995) 155:1151–64.
11. Leonardo SM, De Santis JL, Gehrand A, Malherbe LP, Gauld SB. Expansion of follicular helper T cells in the absence of Treg cells: implications for loss of B-cell anergy. *Eur J Immunol.* (2012) 42:2597–607. doi: 10.1002/eji.201242616
12. Wing JB, Ise W, Kurosaki T, Sakaguchi S. Regulatory T cells control antigen-specific expansion of Tfh cell number and humoral immune responses via the coreceptor CTLA-4. *Immunity* (2014) 41:1013–25. doi: 10.1016/j.immuni.2014.12.006
13. Wildin RS, Smyk-Pearson S, Filipovich AH. Clinical and molecular features of the immunodysregulation, polyendocrinopathy, enteropathy, X linked (IPEX) syndrome. *J Med Genet.* (2002) 39:537–45. doi: 10.1136/jmg.39.8.537
14. Curotto de Lafaille MA, Kutchukhidze N, Shen S, Ding Y, Yee H, Lafaille JJ. Adaptive Foxp3⁺ regulatory T cell-dependent and -independent control of allergic inflammation. *Immunity* (2008) 29:114–26. doi: 10.1016/j.immuni.2008.05.010
15. Chung Y, Tanaka S, Chu F, Nurieva RI, Martinez GJ, Rawal S, et al. Follicular regulatory T cells expressing Foxp3 and Bcl-6 suppress germinal center reactions. *Nat Med.* (2011) 17:983–8. doi: 10.1038/nm.2426
16. Kinnunen T, Chamberlain N, Morbach H, Choi J, Kim S, Craft J, et al. Accumulation of peripheral autoreactive B cells in the absence of functional human regulatory T cells. *Blood* (2013) 121:1595–603. doi: 10.1182/blood-2012-09-457465
17. Wing JB, Sakaguchi S. Foxp3⁺ T(reg) cells in humoral immunity. *Int Immunol.* (2014) 26:61–9. doi: 10.1093/intimm/dxt060
18. Hadaschik EN, Wei X, Leiss H, Heckmann B, Niederreiter B, Steiner G, et al. Regulatory T cell-deficient scurfy mice develop systemic autoimmune features resembling lupus-like disease. *Arthritis Res Ther.* (2015) 17:35. doi: 10.1186/s13075-015-0538-0
19. Wing JB, Sakaguchi S. Multiple treg suppressive modules and their adaptability. *Front Immunol.* (2012) 3:178. doi: 10.3389/fimmu.2012.00178
20. Lim HW, Hillsamer P, Kim CH. Regulatory T cells can migrate to follicles upon T cell activation and suppress GC-Th cells and GC-Th cell-driven B cell responses. *J Clin Invest.* (2004) 114:1640–9. doi: 10.1172/JCI22325
21. Lim HW, Hillsamer P, Banham AH, Kim CH. Cutting edge: direct suppression of B cells by CD4⁺ CD25⁺ regulatory T cells. *J Immunol.* (2005) 175:4180–3. doi: 10.4049/jimmunol.175.7.4180
22. Linterman MA, Pierson W, Lee SK, Kallies A, Kawamoto S, Rayner TF, et al. Foxp3⁺ follicular regulatory T cells control the germinal center response. *Nat Med.* (2011) 17:975–82. doi: 10.1038/nm.2425
23. Wollenberg I, Agua-Doce A, Hernandez A, Almeida C, Oliveira VG, Faro J, et al. Regulation of the germinal center reaction by Foxp3⁺ follicular regulatory T cells. *J. Immunol.* (2011) 187:4553–60. doi: 10.4049/jimmunol.1101328
24. Botta D, Fuller MJ, Marquez-Lago TT, Bachus H, Bradley JE, Weinmann AS, et al. Dynamic regulation of T follicular regulatory cell responses by interleukin 2 during influenza infection. *Nat Immunol.* (2017) 18:1249–60. doi: 10.1038/ni.3837
25. Ritvo PG, Churlaud G, Quiniou V, Florez L, Brimaud F, Fourcade G, et al. (2017). Tfr cells lack IL-2Ralpha but express decoy IL-1R2 and IL-1Ra and suppress the IL-1-dependent activation of Tfh cells. *Sci Immunol* 2:eaa0368. doi: 10.1126/sciimmunol.aan0368
26. Wing JB, Kitagawa Y, Locci M, Hume H, Tay C, Morita T, et al. A distinct subpopulation of CD25⁺ T-follicular regulatory cells localizes in the germinal centers. *Proc Natl Acad Sci USA.* (2017) 114:E6400–9. doi: 10.1073/pnas.1705551114
27. Setoguchi R, Hori S, Takahashi T, Sakaguchi S. Homeostatic maintenance of natural Foxp3(+) CD25(+) CD4(+) regulatory T cells by interleukin (IL)-2 and induction of autoimmune disease by IL-2 neutralization. *J Exp Med.* (2005) 201:723–35. doi: 10.1084/jem.20041982
28. Johnston RJ, Poholek AC, DiToro D, Yusuf I, Eto D, Barnett B, et al. Bcl6 and Blimp-1 are reciprocal and antagonistic regulators of T follicular helper cell differentiation. *Science* (2009) 325:1006–10. doi: 10.1126/science.1175870
29. Ballesteros-Tato A, Leon B, Graf BA, Moquin A, Adams PS, Lund FE, et al. Interleukin-2 inhibits germinal center formation by limiting T follicular helper cell differentiation. *Immunity* (2012) 36:847–56. doi: 10.1016/j.immuni.2012.02.012
30. Johnston RJ, Choi YS, Diamond JA, Yang JA, Crotty S. STAT5 is a potent negative regulator of TFH cell differentiation. *J Exp Med.* (2012) 209:243–50. doi: 10.1084/jem.20111174
31. Bankoti R, Ogawa C, Nguyen T, Emadi L, Couse M, Salehi S, et al. Differential regulation of effector and regulatory T cell function by Blimp1. *Sci Rep.* (2017) 7:12078. doi: 10.1038/s41598-017-12171-3
32. Cretney E, Leung PS, Trezise S, Newman, DM, Rankin LC, Teh CE, et al. Characterization of Blimp-1 function in effector regulatory T cells. *J. Autoimmun.* (2018) 91:73–82. doi: 10.1016/j.jaut.2018.04.003
33. Smigiel KS, Richards E, Srivastava S, Thomas KR, Dudda JC, Klonowski KD, et al. CCR7 provides localized access to IL-2 and defines homeostatically distinct regulatory T cell subsets. *J Exp Med.* (2014) 211:121–36. doi: 10.1084/jem.20131142
34. Sayin I, Radtke AJ, Vella LA, Jin W, Wherry EJ, Buggert M, et al. (2018). Spatial distribution and function of T follicular regulatory cells in human lymph nodes. *J Exp Med.* 215:1531–42 doi: 10.1084/jem.20171940
35. Wallin EF, Jolly EC, Suchanek O, Bradley JA, Espeli M, Jayne DR, et al. Human T-follicular helper and T-follicular regulatory cell maintenance is independent of germinal centers. *Blood* (2014) 124:2666–74. doi: 10.1182/blood-2014-07-585976
36. Attridge K, Wang CJ, Wardzinski L, Kenefeck R, Chamberlain JL, Manzotti C, et al. IL-21 inhibits T cell IL-2 production and impairs Treg homeostasis. *Blood* (2012) 119:4656–64. doi: 10.1182/blood-2011-10-388546
37. Ding Y, Li J, Yang P, Luo B, Wu Q, Zajac AJ, et al. Interleukin-21 promotes germinal center reaction by skewing the follicular regulatory T cell to follicular helper T cell balance in autoimmune BXD2 mice. *Arthritis Rheumatol.* (2014) 66:2601–12. doi: 10.1002/art.38735
38. Jandl C, Liu SM, Canete PF, Warren J, Hughes WE, Vogelzang A, et al. IL-21 restricts T follicular regulatory T cell proliferation through Bcl-6 mediated inhibition of responsiveness to IL-2. *Nat Commun.* (2017) 8:14647. doi: 10.1038/ncomms14647
39. Chang JH, Hu H, Jin J, Puebla-Osorio N, Xiao Y, Gilbert BE, et al. TRAF3 regulates the effector function of regulatory T cells and humoral immune responses. *J Exp Med.* (2013) 211:137–51 doi: 10.1084/jem.20131019
40. Vaeth M, Muller G, Stauss D, Dietz L, Klein-Hessling S, Serfling E, et al. Follicular regulatory T cells control humoral autoimmunity via NFAT2-regulated CXCR5 expression. *J Exp Med.* (2014) 211:545–61. doi: 10.1084/jem.20130604
41. Sebastian M, Lopez-Ocasio M, Metidji A, Rieder SA, Shevach EM, Thornton AM. Helios controls a limited subset of regulatory T cell functions. *J Immunol.* (2016) 196:144–55. doi: 10.4049/jimmunol.1501704
42. Xu L, Huang Q, Wang H, Hao Y, Bai Q, Hu J, et al. The Kinase mTORC1 promotes the generation and suppressive function of follicular regulatory T cells. *Immunity* (2017) 47:538–51.e535. doi: 10.1016/j.immuni.2017.08.011
43. Wu H, Chen Y, Liu H, Xu LL, Teuscher P, Wang S, et al. Follicular regulatory T cells repress cytokine production by follicular helper T cells and optimize IgG responses in mice. *Eur. J. Immunol.* (2016) 46:1152–61. doi: 10.1002/eji.201546094
44. Kawamoto S, Maruya M, Kato LM, Suda W, Atarashi K, Doi Y, et al. Foxp3(+) T cells regulate immunoglobulin a selection and facilitate diversification of bacterial species responsible for immune homeostasis. *Immunity* (2014) 41:152–65. doi: 10.1016/j.immuni.2014.05.016
45. Fu W, Liu X, Lin X, Feng H, Sun L, Li S, et al. Deficiency in T follicular regulatory cells promotes autoimmunity. *J Exp Med.* (2018) 215:815–25. doi: 10.1084/jem.20170901
46. Sage PT, Alvarez D, Godec J, von Andrian UH, Sharpe AH. Circulating T follicular regulatory and helper cells have memory-like properties. *J Clin Invest.* (2014) 124:5191–204. doi: 10.1172/JCI76861
47. Maceiras AR, Almeida SCP, Mariotti-Ferrandiz E, Chaara W, Jebbawi F, Six A, et al. T follicular helper and T follicular regulatory cells have different TCR specificity. *Nat Commun.* (2017) 8:15067. doi: 10.1038/ncomms15067
48. Shulman Z, Gitlin AD, Targ S, Jankovic M, Pasqual G, Nussenzweig MC, et al. T follicular helper cell dynamics in germinal centers. *Science* (2013) 341:673–7. doi: 10.1126/science.1241680

49. Turner JS, Benet ZL, Grigorova IL. Antigen acquisition enables newly arriving B cells to enter ongoing immunization-induced germinal centers. *J Immunol.* (2017) 199:1301–7. doi: 10.4049/jimmunol.1700267
50. Wing K, Onishi Y, Prieto-Martin P, Yamaguchi T, Miyara M, Fehervari Z, et al. CTLA-4 control over Foxp3(+) regulatory T cell function. *Science* (2008) 322:271–5. doi: 10.1126/Science.1160062
51. Sage PT, Paterson AM, Lovitch SB, Sharpe AH. The coinhibitory receptor CTLA-4 controls B cell responses by modulating T follicular helper, T follicular regulatory, and T regulatory cells. *Immunity* (2014) 41:1026–39. doi: 10.1016/j.immuni.2014.12.005
52. Wang CJ, Heuts F, Ovcinnikovs V, Wardzinski L, Bowers C, Schmidt EM, et al. CTLA-4 controls follicular helper T-cell differentiation by regulating the strength of CD28 engagement. *Proc Natl Acad Sci USA.* (2015) 112:524–9. doi: 10.1073/pnas.1414576112
53. Walker LSK, Sansom DM. Confusing signals: recent progress in CTLA-4 biology. *Trends Immunol* (2015) 36: 1–8. doi: 10.1016/j.it.2014.12.001
54. Weber JP, Fuhrmann F, Feist RK, Lahmann A, Al Baz MS, Gentz LJ, et al. ICOS maintains the T follicular helper cell phenotype by down-regulating Kruppel-like factor 2. *J Exp Med.* (2015) 212:217–33. doi: 10.1084/jem.20141432
55. Watanabe M, Fujihara C, Radtke AJ, Chiang YJ, Bhatia S, Germain RN, et al. Co-stimulatory function in primary germinal center responses: CD40 and B7 are required on distinct antigen-presenting cells. *J Exp Med.* (2017) 214:2795–810. doi: 10.1084/jem.20161955
56. Salek-Ardakani S, Choi YS, Rafi-El-Idrissi Benhnia M, Flynn R, Arens R, Shoenberger S, et al. B cell-specific expression of B7-2 is required for follicular Th cell function in response to vaccinia virus. *J Immunol.* (2011) 186:5294–303. doi: 10.4049/jimmunol.1100406
57. Good-Jacobson KL, Song E, Anderson S, Sharpe AH, Shlomchik MJ. CD80 expression on B cells regulates murine T follicular helper development, germinal center B cell survival, and plasma cell generation. *J Immunol.* (2012) 188:4217–25. doi: 10.4049/jimmunol.1102885
58. Walker LS, Wiggett HE, Gaspal FM, Raykundalia CR, Goodall MD, Toellner KM, et al. Established T cell-driven germinal center B cell proliferation is independent of CD28 signaling but is tightly regulated through CTLA-4. *J Immunol.* (2003) 170:91–8. doi: 10.4049/jimmunol.170.1.91
59. De Simone M, Arrigoni A, Rossetti G, Gruarin P, Ranzani V, Politano C, et al. Transcriptional landscape of human tissue lymphocytes unveils uniqueness of tumor-infiltrating T regulatory cells. *Immunity* (2016) 45:1135–47. doi: 10.1016/j.immuni.2016.10.021
60. Tan TG, Mathis D, Benoist C. Singular role for T-BET+CXCR3+ regulatory T cells in protection from autoimmune diabetes. *Proc Natl Acad Sci USA.* (2016) 113:14103–8. doi: 10.1073/pnas.1616710113
61. McCarron MJ, Marie JC. TGF-beta prevents T follicular helper cell accumulation and B cell autoreactivity. *J Clin Invest.* (2014) 124:4375–86. doi: 10.1172/JCI76179
62. Zhao DM, Thornton AM, DiPaolo RJ, Shevach EM. Activated CD4(+)CD25(+) T cells selectively kill B lymphocytes. *Blood* (2006) 107:3925–32. doi: 10.1182/Blood-2005-11-4502
63. Ludwig-Portugall I, Hamilton-Williams EE, Gottschalk C, Kurts C. Cutting edge: CD25+ regulatory T cells prevent expansion and induce apoptosis of B cells specific for tissue autoantigens. *J Immunol.* (2008) 181:4447–51. doi: 10.4049/jimmunol.181.7.4447
64. Laidlaw BJ, Lu Y, Amezquita RA, Weinstein JS, Vander Heiden JA, Gupta NT, et al. Interleukin-10 from CD4(+) follicular regulatory T cells promotes the germinal center response. *Sci Immunol.* (2017) 2:eaan4767. doi: 10.1126/sciimmunol.aan4767
65. Cai G, Nie X, Zhang W, Wu B, Lin J, Wang H, et al. A regulatory role for IL-10 receptor signaling in development and B cell help of T follicular helper cells in mice. *J Immunol.* (2012) 189:1294–302. doi: 10.4049/jimmunol.1102948
66. Xie MM, Dent AL. Unexpected help: follicular regulatory T cells in the germinal center. *Front Immunol.* (2018) 9:1536. doi: 10.3389/fimmu.2018.01536
67. Sage PT, Ron-Harel N, Juneja VR, Sen DR, Maleri S, Sungnak W, et al. Suppression by TFR cells leads to durable and selective inhibition of B cell effector function. *Nat Immunol.* (2016) 17:1436–46. doi: 10.1038/ni.3578
68. Fonseca VR, Agua-Doce A, Maceiras AR, Pierson W, Ribeiro F, Romao VC, et al. Human blood Tfr cells are indicators of ongoing humoral activity not fully licensed with suppressive function. *Sci Immunol.* (2017) 2:eaan1487. doi: 10.1126/sciimmunol.aan1487
69. Dhaeze T, Peelen E, Hombrouck A, Peeters L, Van Wijmeersch B, Lemkens N, et al. Circulating follicular regulatory T cells are defective in multiple sclerosis. *J Immunol.* (2015) 195:832–40. doi: 10.4049/jimmunol.1500759
70. Miyara M, Yoshioka Y, Kitoh A, Shima T, Wing K, Niwa A, et al. Functional delineation and differentiation dynamics of human CD4+ T cells expressing the FoxP3 transcription factor. *Immunity* (2009) 30:899–911. doi: 10.1016/j.immuni.2009.03.019
71. Liu C, Wang D, Lu S, Xu Q, Zhao L, Zhao J, et al. Increased circulating follicular treg cells are associated with lower levels of autoantibodies in patients with rheumatoid arthritis in stable remission. *Arthritis Rheumatol.* (2018) 70:711–21. doi: 10.1002/art.40430
72. Cuadrado E, van den Biggelaar M, de Kivit S, Chen YY, Slot M, Doubal I, et al. Proteomic analyses of human regulatory T cells reveal adaptations in signaling pathways that protect cellular identity. *Immunity* (2018) 48:1046–59. doi: 10.1016/j.immuni.2018.04.008
73. Lim HW, Broxmeyer HE, Kim CH. Regulation of trafficking receptor expression in human forkhead box P3+ regulatory T cells. *J Immunol.* (2006) 177:840–51. doi: 10.4049/jimmunol.177.2.840
74. Canavan JB, Scotta C, Vossenkamper A, Goldberg R, Elder MJ, Shoval I, et al. Developing *in vitro* expanded CD45RA+ regulatory T cells as an adoptive cell therapy for Crohn's disease. *Gut* (2016) 65:584–94. doi: 10.1136/gutjnl-2014-306919
75. Fonseca VR, Romao VC, Agua-Doce A, Santos M, Lopez-Presa D, Ferreira AC, et al. The ratio of blood T follicular regulatory cells to T follicular helper cells marks ectopic lymphoid structure formation while activated follicular helper T cells indicate disease activity in primary Sjogren's syndrome. *Arthritis Rheumatol.* (2018) 70:774–84. doi: 10.1002/art.40424
76. Wang L, Qiu J, Yu L, Hu X, Zhao P, Jiang Y. Increased numbers of CD5+CD19+CD1dhighIL-10+ Bregs, CD4+Foxp3+ Tregs, CD4+CXCR5+Foxp3+ follicular regulatory T (TFR) cells in CHB or CHC patients. *J Transl Med.* (2014) 12:251. doi: 10.1186/s12967-014-0251-9
77. Chen X, Li W, Zhang Y, Song X, Xu L, Xu Z, et al. Distribution of peripheral memory T follicular helper cells in patients with *Schistosomiasis Japonica*. *PLoS Negl Trop Dis.* (2015) 9:e0004015. doi: 10.1371/journal.pntd.0004015
78. Miles B, Miller SM, Folkvord JM, Kimball A, Chamanian M, Meditz AL, et al. Follicular regulatory T cells impair follicular T helper cells in HIV and SIV infection. *Nat Commun.* (2015) 6:8608. doi: 10.1038/ncomms9608
79. Romão VC, Agua-Doce A, Fonseca JE, Graca L. T follicular regulatory cells are decreased in established treated rheumatoid arthritis patients with active disease. *Arthritis Rheumatol.* (2018). doi: 10.1002/art.40586. [Epub ahead of print].
80. Xu B, Wang S, Zhou M, Huang Y, Fu R, Guo C, et al. The ratio of circulating follicular T helper cell to follicular T regulatory cell is correlated with disease activity in systemic lupus erythematosus. *Clin Immunol.* (2017) 183:46–53. doi: 10.1016/j.clim.2017.07.004
81. Cunill V, Clemente A, Lanio N, Barcelo C, Andreu V, Pons J, et al. Follicular T cells from smB(-) common variable immunodeficiency patients are skewed toward a Th1 phenotype. *Front Immunol.* (2017) 8:174. doi: 10.3389/fimmu.2017.00174
82. Vaeth M, Eckstein M, Shaw PJ, Kozhaya L, Yang J, Berberich-Siebelt F, et al. Store-operated Ca(2+) entry in follicular T cells controls humoral immune responses and autoimmunity. *Immunity* (2016) 44:1350–64. doi: 10.1016/j.immuni.2016.04.013
83. Ma CS, Wong N, Rao G, Avery DT, Torpy J, Hambridge T, et al. Monogenic mutations differentially affect the quantity and quality of T follicular helper cells in patients with human primary immunodeficiencies. *J Allergy Clin Immunol.* (2015) 136:993–1006.e1001. doi: 10.1016/j.jaci.2015.05.036

84. Guo Z, Liang H, Xu Y, Liu L, Ren X, Zhang S, et al. The role of circulating T follicular helper cells and regulatory cells in non-small cell lung cancer patients. *Scand J Immunol.* (2017) 86:107–12. doi: 10.1111/sji.12566
85. Cha Z, Qian G, Zang Y, Gu H, Huang Y, Zhu L, et al. Circulating CXCR5+CD4+ T cells assist in the survival and growth of primary diffuse large B cell lymphoma cells through interleukin 10 pathway. *Exp Cell Res.* (2017) 350:154–60. doi: 10.1016/j.yexcr.2016.11.017
86. Gu-Trantien C, Loi S, Garaud S, Equeter C, Libin M, de Wind A, et al. CD4(+) follicular helper T cell infiltration predicts breast cancer survival. *J Clin Invest.* (2013) 123:2873–92. doi: 10.1172/JCI67428
87. Gu-Trantien C, Migliori E, Buisseret L, de Wind A, Brohee S, Garaud S, et al. CXCL13-producing TFH cells link immune suppression and

adaptive memory in human breast cancer. *JCI Insight* (2017). 2:91487. doi: 10.1172/jci.insight.91487

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2018 Wing, Tekgüç and Sakaguchi. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



T Cell/B Cell Collaboration and Autoimmunity: An Intimate Relationship

Lina Petersone, Natalie M. Edner, Vitalijs Ovcinnikovs, Frank Heuts, Ellen M. Ross, Elisavet Ntavli, Chun J. Wang and Lucy S. K. Walker*

Division of Infection and Immunity, Institute of Immunity and Transplantation, University College London, London, United Kingdom

OPEN ACCESS

Edited by:

Georgia Fousteri,
San Raffaele Hospital (IRCCS), Italy

Reviewed by:

George Kassiotis,
Naval Medical Research Center,
United States
Tri Giang Phan,
Garvan Institute of Medical Research,
Australia

*Correspondence:

Lucy S. K. Walker
lucy.walker@ucl.ac.uk

Specialty section:

This article was submitted to
T Cell Biology,
a section of the journal
Frontiers in Immunology

Received: 05 June 2018

Accepted: 06 August 2018

Published: 27 August 2018

Citation:

Petersone L, Edner NM,
Ovcinnikovs V, Heuts F, Ross EM,
Ntavli E, Wang CJ and Walker LSK
(2018) T Cell/B Cell Collaboration and
Autoimmunity: An Intimate
Relationship. *Front. Immunol.* 9:1941.
doi: 10.3389/fimmu.2018.01941

Co-ordinated interaction between distinct cell types is a hallmark of successful immune function. A striking example of this is the carefully orchestrated cooperation between helper T cells and B cells that occurs during the initiation and fine-tuning of T-cell dependent antibody responses. While these processes have evolved to permit rapid immune defense against infection, it is becoming increasingly clear that such interactions can also underpin the development of autoimmunity. Here we discuss a selection of cellular and molecular pathways that mediate T cell/B cell collaboration and highlight how *in vivo* models and genome wide association studies link them with autoimmune disease. In particular, we emphasize how CTLA-4-mediated regulation of CD28 signaling controls the engagement of secondary costimulatory pathways such as ICOS and OX40, and profoundly influences the capacity of T cells to provide B cell help. While our molecular understanding of the co-operation between T cells and B cells derives from analysis of secondary lymphoid tissues, emerging evidence suggests that subtly different rules may govern the interaction of T and B cells at ectopic sites during autoimmune inflammation. Accordingly, the phenotype of the T cells providing help at these sites includes notable distinctions, despite sharing core features with T cells imparting help in secondary lymphoid tissues. Finally, we highlight the interdependence of T cell and B cell responses and suggest that a significant beneficial impact of B cell depletion in autoimmune settings may be its detrimental effect on T cells engaged in molecular conversation with B cells.

Keywords: follicular helper T cells (T_{fh}), B cells, germinal center, autoimmunity, costimulation, CD28, CTLA-4, immunotherapy

INTRODUCTION

Effective collaboration between T and B cells is a central tenet of protective immunity. Such interactions underlie the development of optimal affinity-matured antibody responses that are required for host defense, permitting the rapid neutralization of bacterial toxins and blockade of viral cell entry. Over the last decade however, it has become apparent that T cell/B cell collaboration also underpins the development of many autoimmune responses leading to undesirable sequelae. Thus, many of the cellular and molecular pathways familiar to us in the context of effective immunity are also implicated in the development of autoimmunity.

In this review we highlight the interdependence of T cell and B cell responses, both in the initiation of humoral immunity and in the context of immune memory. We then home in on the

pathways supporting T cell/B cell collaboration and discuss how costimulatory signals orchestrate the chemokine receptor modulation that drives T cell localization to the T-B border and the altered motility that promotes follicular entry. The importance of SLAM family members in stabilizing adhesive interactions between T and B cells is considered, as is the role of cytokines that support or hinder the emergence of T cell help for the B cell response. Next we examine the early work linking follicular helper T cell (Tfh) differentiation to the development of autoimmunity in mice and describe how this prompted a wave of interest in the analysis of blood-borne Tfh-like cells in human autoimmunity. We illustrate how many of the pathways considered earlier are linked to human autoimmunity by probing GWAS datasets for 10 selected autoimmune diseases.

Throughout the review we focus in particular on the Tfh cell subset that enter B cell follicles to support germinal center (GC) formation. However, it is important to note that interactions between B cells and non-Tfh subsets may also play roles in promoting autoimmunity. A recent exciting development in this regard is covered in our final section on T Cell/B Cell Collaboration Outside Secondary Lymphoid Tissues where we discuss the identification of “peripheral helper” T cells that lack bona fide Tfh markers yet appear to provide help to B cells at sites of autoimmune inflammation. Finally, we close the article by discussing the potential to interrupt T cell/B cell collaboration in autoimmune settings by therapeutic B cell depletion.

INTERDEPENDENCE OF T CELL AND B CELL RESPONSES

Implicit in the concept of T cell help for B cells is a notion of directionality, implying that T cells are the providers of help and B cells the recipients. However, it has become clear that the reality is far more equitable, with sequential inputs required from both cell types for a successful overall outcome. This is elegantly demonstrated by the molecular underpinnings of the germinal center response, which relies on tightly regulated bi-directional interactions between follicular helper T cells (Tfh) and B cells.

Tfh cell differentiation is a highly complex multistage endeavor [reviewed in (1)], and B cells play an integral role in this process from the moment Tfh cell precursors first interact with B cells at the follicular border in spleen or interfollicular region in lymph nodes (2, 3) and throughout the GC reaction. In the absence of cognate B cells, Tfh precursors expressing Bcl6 (the master transcription factor for Tfh differentiation) fail to assume a mature Tfh cell phenotype within the follicle (3). The maintenance of Tfh cells requires sustained antigenic stimulation and B cells represent the key antigen presenting cell type during the GC reaction (4, 5). Moreover, there is a positive correlation between Tfh cell and GC B cell numbers in GC, emphasizing the intimate functional relationship between the two cell subsets (4, 6).

When it comes to memory responses, T cells play a clear role in the emergence of memory B cells via the GC reaction, and it appears that the inverse is also true, with B cells

actively supporting the efficient generation or maintenance of T cell memory (7). Elegant experiments revealed a key role for memory B cells in presenting antigen to memory Tfh cells to drive Bcl6 re-expression (8), and the location of memory Tfh cells in B cell follicles (9, 10) makes them ideally placed for such contacts. In addition to cognate interaction, the role of B cells in T cell memory may include the provision of costimulatory ligands, as well as their contribution to the structural organization and architecture that supports immune responses.

PATHWAYS SUPPORTING T CELL/B CELL COLLABORATION

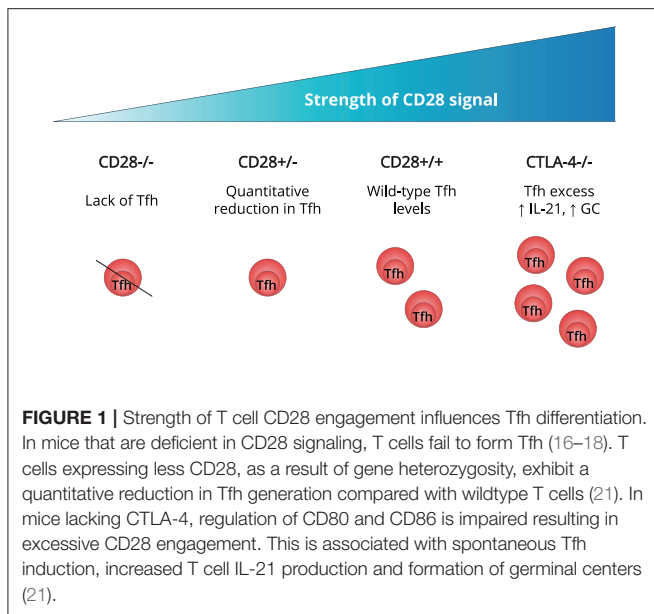
Several key pathways regulating T cell/B cell collaboration have been identified over the years (11), and we highlight a number of examples below.

CD40/CD40L

CD40 and CD40L have long been recognized as key players in humoral immunity and are essential for GC formation (11–13). Blockade of CD40L signaling during an ongoing GC reaction was shown to abrogate the response, emphasizing the need for continuous CD40-CD40L interactions throughout the GC lifespan (14). Clinical studies identified mutations in CD40L as a common cause for human genetic immunodeficiency X-linked hyper-IgM syndrome, where patients presented with impaired GC development emphasizing the importance of T cell/B cell collaboration in the pre-GC stages of adaptive immune responses (11, 15).

CD28/CTLA-4

Experiments in the late 1990's established that CD28 signaling was required for CD4 T cells to upregulate CXCR5 and migrate into B cell follicles (16), explaining the defect in GC formation in mice lacking CD28 (17) or its ligands (18). CXCR5 induction permits responsiveness to CXCL13 expressed by stromal cells in the follicle and, in association with downregulation of CCR7 (19), guides T cell follicular migration. The G-protein-coupled receptor S1PR2 appears to cooperate with CXCR5 to ensure localization and retention of Tfh at the GC site (20). The amount of CD28 engagement directly influences Tfh differentiation since T cells heterozygous for CD28 showed reduced Tfh induction despite normal activation (**Figure 1**). The CD28 pathway is regulated by CTLA-4 which binds to the same ligands, CD80 and CD86, but with higher affinity than CD28. Although widely credited with imparting a negative signal, in our view the available evidence does not support this idea and instead suggests that CTLA-4 regulates CD28 engagement by competing for and downregulating their shared ligands (22, 23). The CTLA-4 pathway restricts the formation of Tfh by limiting T cell CD28 engagement (21) and CTLA-4 expression in the regulatory T cell compartment is essential for this process (24, 25). Accordingly, deficiency or blockade of CTLA-4 in mice leads to hyper-engagement of CD28, overproduction of Tfh and spontaneous GC formation (21). CD28 is also required for the development of the follicular



regulatory T cells (Tfr) that negatively regulate the GC response (26) (for recent reviews of Tfr please see Wing et al. (27), Fazilleau and Aloulou (28) and Xie and Dent (29) in this collection).

OX40

The ability of CD28 to promote Tfh development may reflect its capacity to upregulate secondary costimulatory receptors such as OX40 and ICOS. CD28 engagement triggers T cell OX40 upregulation (16) and ligation of OX40 in turn promotes CXCR5 expression (30). Mice expressing OX40L constitutively on dendritic cells showed increased numbers of CD4 T cells in their B cell follicles (31) and conversely deficiency (32) or blockade (33) of OX40 reduced Tfh numbers after viral challenge. Importantly, B cell expression of OX40L has also been shown to support Tfh development (34).

Despite the above, the involvement of OX40 in Tfh differentiation remains controversial; indeed in one study engagement of OX40 was shown to impair Tfh development by promoting expression of Blimp-1 (35) which can inhibit Bcl6 and extinguish the Tfh programme (36). Similarly, in the context of *Listeria monocytogenes* infection, mice lacking OX40 showed intact Tfh differentiation, and treatment of wildtype mice with agonistic anti-OX40 antibodies expanded effector T cells at the expense of Tfh (37). Thus the involvement of OX40 may be context dependent, with strain-specific and site-specific differences being noted in one study (38). It remains possible that OX40 stimulates the survival or expansion of all differentiated T cells rather than instructing the Tfh differentiation process *per se*.

ICOS

ICOS is known to be required for the GC response (39–42) and its engagement promotes the differentiation (43) and maintenance

(44) of Tfh cells. The level of ICOS upregulation on T cells undergoing activation *in vivo* is tightly coupled to the level of CD28 engagement (21) consistent with the idea that CD28 may promote GC formation via the ICOS pathway. ICOS is superior to CD28 in its capacity to activate phosphoinositide 3-kinase which is known to be required for Tfh cell differentiation and GC formation (6, 45). It has been suggested that ICOS can substitute for CD28 in later phases of the Tfh response (46) although the timing may be critical since extinguishing CD28 at the time of OX40 induction (using OX40-Cre CD28-floxed mice) showed the response was still CD28-dependent at this stage (47, 48). B cells may be an important source of ICOSL since mice lacking B cell-expression of this molecule exhibit significantly reduced Tfh and GC B cell numbers in response to peptide immunization (49, 50). Intriguingly this may reflect a role for ICOSL on bystander (non-cognate) B cells which engages ICOS on T cells approaching the T-B border, promoting their motility and hastening their follicular entry and subsequent Tfh maturation (51). ICOS signaling downregulates the transcription factor Klf2 in both mouse and human T cells and this is critical for ensuring follicular localization of Tfh by keeping CXCR5 high but CCR7, CD62L, PSGL-1, and S1PR1 low (44). Mirroring the findings in murine models, humans with ICOS deficiency show reduced blood Tfh cell frequencies and defects in GC and memory B cell formation (52, 53).

SLAM Family Members

During a GC reaction, T and B cells are required to repeatedly engage with each other to facilitate interactions between the receptor/ligand pairs described above. At the T-B border, early interactions between antigen-specific T and B cells are long-lived, while within GC, most cognate Tfh/GC B cell interactions last less than 5 min, but are associated with extensive surface contacts (54, 55). These interactions are stabilized by expression of signal lymphocyte activation molecule (SLAM) family receptors Ly108 and CD84 and SLAM-associated protein (SAP) (56, 57). The importance of these molecules is highlighted by SAP-deficient mice, where Tfh cell differentiation is impaired leading to profound defects in formation of GC, long-lived plasma cells and memory B cells (58–61). Similar observations have been made in X-linked lymphoproliferative disease patients with SAP-deficiency (62).

Cytokines

IL-2 is a powerful inhibitor of Tfh differentiation (43, 63) by virtue of its STAT5-dependent induction of Blimp-1 (43, 64). Intriguingly, it has been shown that activated dendritic cells in the outer T zone use CD25 expression to quench T cell derived IL-2 thereby generating a microenvironment that favors Tfh formation (65). Tfh differentiation is also influenced by other cytokines, most notably IL-6 in mice (66) and IL-12 in humans (67, 68). Intravital imaging studies have revealed that cognate interactions with GC B cells induce Ca^{2+} -dependent co-expression of IL-21 and IL-4 in Tfh (69). These cytokines further promote GC B cell responses, providing a positive feedback loop between Tfh and GC B cells.

T CELL/B CELL COLLABORATION IN AUTOIMMUNITY

Widespread recognition of the importance of T cell/B cell collaboration in driving immune-mediated pathology came from a landmark paper in 2009 (70) linking overproduction of Tfh with systemic autoimmunity. This work focused on sanroque mice which have a mutation in the E3 ubiquitin ligase Roquin-1 that regulates mRNA stability and is required for appropriate repression of ICOS expression. Mice with the *Roquin* mutation exhibited high ICOS expression, excessive Tfh formation and lupus-like pathology, however this was abolished if the mice were rendered SAP-deficient, consistent with a critical role for T cell/B cell collaboration in driving this pathology. It was subsequently shown that the *Roquin* mutation dramatically increased progression to type 1 diabetes (T1D) in a TCR transgenic mouse model (71). In a separate mouse model, microarray analysis of T cells responding to pancreatic antigen revealed a striking signature for Tfh differentiation, and cells with a Tfh phenotype showed an enhanced capacity to induce diabetes upon adoptive transfer (72). SAP dependent T cell/B cell interactions have been shown to be essential in the K/BxN model of arthritis (73), where a role for gut microbiota in promoting disease via Tfh induction has been identified (74). A separate study revealed that collagen-induced arthritis could be ameliorated by T cell specific CXCR5 deficiency consistent with the potential involvement of Tfh (75). Findings from mouse models prompted investigation of cells with a Tfh-like phenotype in a wide variety of disease settings in humans, leading to the appreciation that these cells are overrepresented in multiple autoimmune diseases including systemic lupus erythematosus (SLE), Sjögren's syndrome, T1D, myasthenia gravis, rheumatoid arthritis (RA) and multiple sclerosis (MS) (76–78).

The exact provenance of blood-borne cells with a Tfh phenotype has been the subject of much debate. Elegant intravital imaging revealed that while Tfh readily move between GC they only rarely enter the circulation (79). It is widely recognized that Tfh have a circulating memory counterpart (80–83), however expression of many Tfh markers is reduced in the circulation (84, 85) with CXCR5 being least affected (4). Blood-borne CD4+CXCR5+ cells have been shown to be superior at supporting B cell antibody production and class-switching *in vitro* compared to their CD4+CXCR5- counterparts (86–90). Importantly, CXCR5+ cells can be found in the blood of SAP-deficient mice and humans, consistent with the idea that they arise prior to T cell differentiation into mature Tfh within GC (89). Despite their controversial origin and likely heterogeneity, it has become clear that upon antigen exposure circulating Tfh-phenotype cells can migrate to secondary lymphoid tissue and participate in GC reactions suggesting they represent a bona fide functional memory subset (91).

There are many possible explanations for the observed elevation in Tfh-like cells in autoimmune settings. In some cases, this may be secondary to generalized immune activation associated with disease. However, Tfh changes can be detected prior to the onset of overt disease in children at risk of T1D (92), and insulin-specific T cells are enriched for a CXCR5+

Tfh precursor population in children who have only recently developed islet autoantibodies (93). The blood Tfh signature is frequently linked to disease activity (76, 78), and successful treatment of SLE has been shown to decrease Tfh while numbers of Th1 and Th2 cells remain unaltered (94). Persistent antigen has been suggested to favor Tfh differentiation and maintenance (4, 95), so continuous availability of tissue antigen could potentially support this response in chronic autoimmune conditions.

The strongest genetic association with autoimmunity maps to the HLA region (96), consistent with its role in presenting the TCR ligands that drive pathogenic and regulatory (97) T cell responses. Interestingly, other genes conferring susceptibility to autoimmunity in humans include many candidates associated with T cell/B cell collaboration. Accordingly, in genome-wide association studies (GWAS) from ten selected autoimmune conditions (T1D, RA, juvenile idiopathic arthritis, autoimmune thyroid diseases, vitiligo, alopecia areata, SLE, MS, primary biliary cirrhosis, celiac disease), polymorphisms in several genes integral for T cell/B cell co-operation bear significant associations with disease susceptibility (Figure 2). These genes are highlighted on the basis of their relevance to T cell/B cell collaboration, however it should be noted that many are also likely to influence T cell interactions with other cell types, such as dendritic cells. A selection of these is discussed below.

Costimulatory Molecules

The *CD28*, *CTLA4*, and *ICOS* genes are located within a 300 kb region on human chromosome 2 and likely arose from sequential gene duplication (99). Variation at this locus is associated with autoimmunity (100) and blockade of CD28 signaling with CTLA-4-Ig fusion protein is a recognized treatment strategy in a number of autoimmune disease settings (101). As mentioned above, autoimmunity in sanroque mice is associated with de-repression of ICOS mRNA, and dysregulated ICOS expression is also believed to underlie the increase in Tfh and autoimmune phenotype seen in the Sle1 lupus-prone mouse model (102). The genes encoding the ligands for these receptors, *CD80*, *CD86*, and *ICOSLG*, are also associated with autoimmunity (Figure 2), consistent with the need to tightly control the core pathways that control the induction of T cell help. *CD40*, which provides an essential pathway for GC B cells to perceive T cell help, is implicated in multiple autoimmune diseases (103) as is *DBC1* which regulates its downstream signaling (104). *OX40L* contributes to pathology in a mouse model of SLE (34) and polymorphisms in *OX40L* (*TNFSF4*) are associated with several diseases where humoral immunity is known to be perturbed including SLE and RA, leading to the investigation of this pathway as a therapeutic target (105).

Cytokines

Cytokines are important regulators of the GC response, and many of the key cytokines implicated in shaping Tfh and GC B cell differentiation are associated with autoimmune susceptibility. *IL2RA*, which encodes the high affinity subunit for the IL-2-receptor shows one of the strongest associations with T1D outside of the HLA region (106), and as discussed above, IL-2 signaling potently inhibits Tfh differentiation. The *IL2* and *IL21* genes located next to each other on human chromosome 4

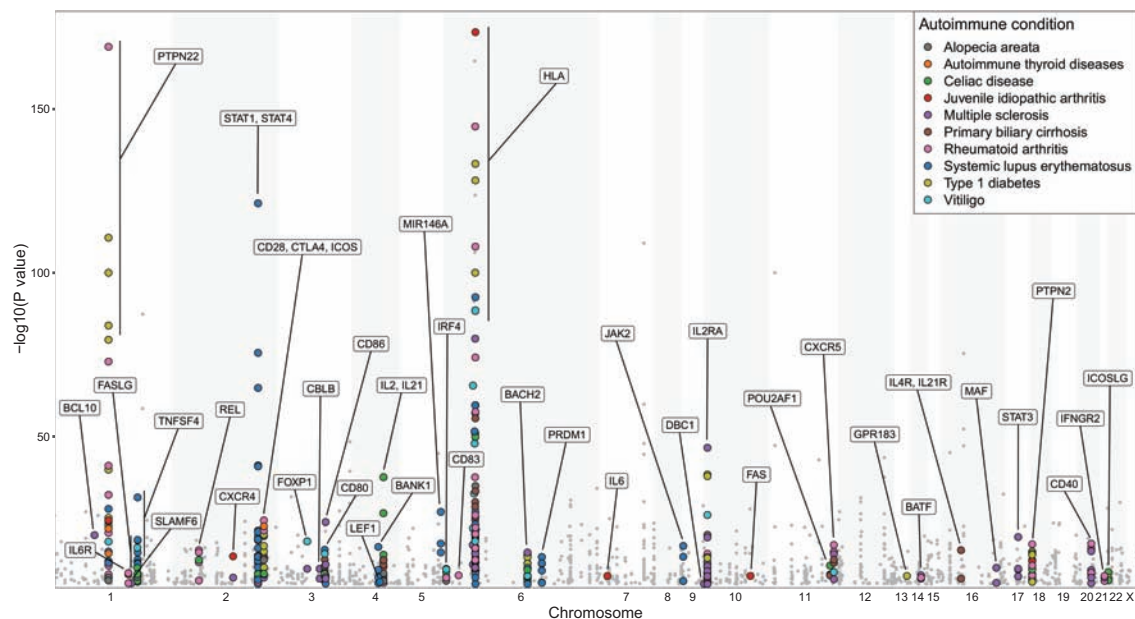


FIGURE 2 | Genes involved in regulating T cell/B cell collaboration are associated with autoimmunity. Manhattan plot of GWAS analysis for 10 selected autoimmune diseases: alopecia areata, autoimmune thyroid diseases (Hashimoto's thyroiditis and Graves' disease), celiac disease, juvenile idiopathic arthritis, MS, primary biliary cirrhosis, RA, SLE, T1D, and vitiligo. Statistical strength of association ($-\log_{10}P$) is plotted against genomic position. Single nucleotide polymorphisms (SNPs) in genes with known function in regulating T cell/B cell collaboration are marked with colored circles; other SNPs are marked with gray dots. More than 60% of the highlighted genes are implicated in more than one of the 10 selected autoimmune diseases. The NHGRI-EBI Catalog of published genome-wide association studies (98) was used to generate this figure (catalog version: gwas_catalog_v1.0.2-associations_e92_r2018-05-12; access date 17.05.2018). Only SNP traits with a p -value of $<1.0 \times 10^{-5}$ in the overall (initial GWAS + replication) population are included in this catalog. For clarity, the y-axis scale used in this figure excludes HLA-DRB1 SNP associations with $-\log_{10}P$ values of 299, 250 (x2), 185.4 in RA and 224.4, 206, 183 in MS, and an insulin SNP association with $-\log_{10}P$ of 185 in T1D. This figure only serves illustrative purposes and no direct comparisons should be made between associations taken from different studies.

(107), and *IL4RA* and *IL21RA* on chromosome 16 (108) also bear a strong association with autoimmunity, potentially reflecting the key roles of IL-21 and IL-4 in orchestrating collaboration between Tfh and B cells within GC (109, 110). Also highlighted by GWAS are *IL6*, *IL6R*, and *BANK1* which controls IL-6 secretion (111). While IL-12 is considered to be the major cytokine driving Tfh formation in humans (67, 68), this differentiation fate can also be promoted by IL-6. The demonstration that plasmablast-derived IL-6 can promote Tfh differentiation, in a manner that can be inhibited by treatment with the anti-IL-6R antibody tocilizumab (112), highlights a further positive feedback loop between Tfh and B cells.

ADDITIONAL GENES LINKED TO T CELL/B CELL COLLABORATION

Other autoimmune-susceptibility genes featured in **Figure 2** include the protein tyrosine phosphatase *PTPN22*, which controls the number and activity of Tfh cells (113), and *PTPN2*, deficiency of which leads to increased Tfh cells, GC and autoimmune pathology (114). The chemokine receptors *CXCR5* and *CXCR4*, which play integral roles in regulating cell distribution across GC and facilitating Tfh and GC B cell interactions, are also highlighted (19, 115). The

GWAS data also highlight *Gpr183*, the gene encoding the $7\alpha,25$ -dihydroxycholesterol receptor EBI-2, which must be downregulated for appropriate B cell positioning in GC (116, 117). Indeed forced expression of EBI-2 was shown to diminish the GC response and instead direct B cells to extrafollicular sites (117) while transduction of T cells with an EBI-2 expression vector impaired their capacity to localize to GC (118). Another gene product associated with autoimmunity in this dataset is *SLAMF6*, which co-operates with SAP to promote T cell/B cell adhesion and is essential for formation of functional GC (119). Importantly, in addition to surface molecules and soluble factors, the GWAS data also draw attention to a number of transcription factors associated with the GC response including *BATF*, *IRF4*, *Maf*, *Bob1* (*Pou2af1*), *Rel* and *Blimp-1* (*Prdm1*) (36, 120–125), further highlighting the link between T and B cell interactions and autoimmune susceptibility.

T CELL/B CELL COLLABORATION OUTSIDE SECONDARY LYMPHOID TISSUES

Development of tertiary lymphoid structures is frequently seen in chronically inflamed tissues (126), and T cell/B cell collaboration at ectopic sites has been suggested to fuel ongoing

autoimmunity (127). Recent findings suggest T cells providing B cell help outside secondary lymphoid organs may bear a distinct phenotype; accordingly Rao et al. described a PD-1^{hi}CXCR5⁺CD4⁺ “peripheral helper” T cell population in the synovium of patients with RA which lacked Bcl6 but expressed IL-21, CXCL13, ICOS, and Maf (128). These cells actively promoted memory B cell differentiation into plasma cells *in vitro* and were located adjacent to B cells both inside and outside synovial lymphoid aggregates.

Similarly, in a murine model of airway inflammation, T cells interacting with B cells in the lung exhibited a CXCR5⁺Bcl6⁺ phenotype despite possessing high B cell helper potential, likely *via* their expression of CD40L, IL-21, and IL-4 (129). Remarkably, around 40% of lung-infiltrating B cells in this model showed a GC phenotype implying effective T cell/B cell collaboration, even though the cells were present in loose aggregates rather than well-organized structures.

The relationship of peripheral helper T cells to Tfh cells is currently unclear. However, one study documenting CXCR5⁺BCL6⁺CXCL13⁺ T cells in rheumatoid synovial fluid postulated that these may derive from Tfh cells undergoing progressive differentiation, and loss of CXCR5 and Bcl6 in the synovium (130). The provenance of peripheral helper T cells remains an important question for future clarification.

INTERRUPTING T CELL/B CELL COLLABORATION BY B CELL DEPLETION

Since autoimmunity may arise through over-exuberant T and B cell interactions leading to autoantibody production, depletion of the B cell population has been explored as a treatment strategy. Surprisingly, this has only a moderate effect on serum autoantibody levels, which does not correlate with efficacy, implying an alternative mechanism underlies the beneficial impact (131, 132). Given the interdependence of Tfh and B cell responses highlighted above, one possibility is that B cell depletion affects Tfh cells. Indeed, it has been shown in mice that deletion of GC B cells substantially impairs Tfh homeostasis (4, 133).

In human studies, Xu et al. reported a significant reduction in circulating Tfh frequencies and serum IL-21 levels following B cell depletion with rituximab in patients with T1D, emphasizing Tfh and B cell interdependence in this disease setting (134). Similarly, the elevation in circulating Tfh seen in individuals with Sjögren's syndrome was shown to be normalized by B cell depletion (135).

However, a study by Wallin et al. found no reduction in Tfh numbers in lymph nodes and blood from patients treated with

rituximab prior to kidney transplantation (136). This finding may reflect “setting-dependent” roles for B cells in Tfh cell maintenance in humans. Of note, this study identified Tfh cells using CD57 expression which was initially reported to mark GC-resident functionally mature Tfh (137, 138), but was subsequently shown to be expressed by less than a third of GC-resident Tfh cells (139). Therefore, investigating the dynamics of CD57⁺ Tfh cells may be of interest here.

More recently, B cell depletion with ocrelizumab, a humanized anti-CD20 antibody, has been shown to slow disease progression in patients with some forms of MS when compared to placebo or interferon beta-1a treatment (140, 141). Whether B cell depletion impacts Tfh homeostasis in this disease setting is currently unclear, however treatment has been associated with a decrease in cerebrospinal fluid (CSF) T cells as well as B cells and a reduction in CSF levels of the chemokine CXCL13 (142) which can be produced by Tfh cells. Overall, effects on Tfh homeostasis may offer an additional explanation for the efficacy of B cell depletion in certain settings.

CONCLUSION

Cooperation between T cells and B cells has been fine-tuned by evolutionary pressures to optimize rapid immune defense. These interactions ensure successful long-term immunity, exemplified by the development of effective T cell and B cell memory. Given that chronic autoimmune diseases may be sustained by the perpetuation, rather than initiation, of self-directed immune responses, the bi-directional interaction between T and B cells may be key to this and may therefore constitute an important therapeutic target.

AUTHOR CONTRIBUTIONS

LP wrote and edited the manuscript and designed figures. NE designed figures and reviewed and edited the manuscript. VO, FH, ER, EN, and CW reviewed and edited the manuscript. LW conceptualized, wrote and edited the manuscript. All authors approved the final version of the manuscript.

ACKNOWLEDGMENTS

The work of the authors is supported by the Medical Research Council, Diabetes UK and The Rosetrees Trust. The authors have received funding from the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No 675395.

REFERENCES

- Crotty S. T follicular helper cell differentiation, function, and roles in disease. *Immunity* (2014) 41:529–42. doi: 10.1016/j.immuni.2014.10.004
- Coffey F, Alabyev B, Manser T. Initial clonal expansion of germinal center B cells takes place at the perimeter of follicles. *Immunity* (2009) 30:599–609. doi: 10.1016/j.immuni.2009.01.011
- Kerfoot SM, Yaari G, Patel JR, Johnson KL, Gonzalez DG, Kleinstein SH, et al. Germinal center B cell and T follicular helper cell development initiates in the interfollicular zone. *Immunity* (2011) 34:947–60. doi: 10.1016/j.immuni.2011.03.024
- Baumjohann D, Preite S, Reboldi A, Ronchi F, Ansel KMM, Lanzavecchia A, et al. Persistent antigen and germinal center B cells sustain T

- follicular helper cell responses and phenotype. *Immunity* (2013) 38:596–605. doi: 10.1016/j.immuni.2012.11.020
5. Deenick EK, Chan A, Ma CS, Gatto D, Schwartzberg PL, Brink R, et al. Follicular helper T cell differentiation requires continuous antigen presentation that is independent of unique B cell signaling. *Immunity* (2010) 33:241–53. doi: 10.1016/j.immuni.2010.07.015
6. Rolf J, Bell SE, Kovacs D, Janas ML, Soond DR, Webb LMC, et al. Phosphoinositide 3-kinase activity in T cells regulates the magnitude of the germinal center reaction. *J Immunol.* (2010) 185:4042–52. doi: 10.4049/jimmunol.1001730
7. Whitmire JK, Asano MS, Kaech SM, Sarkar S, Hannum LG, Shlomchik MJ, et al. Requirement of B cells for generating CD4+ T cell memory. *J Immunol.* (2009) 182:1868–76. doi: 10.4049/jimmunol.0802501
8. Ise W, Inoue T, McLachlan JB, Kometani K, Kubo M, Okada T, et al. Memory B cells contribute to rapid Bcl6 expression by memory follicular helper T cells. *Proc Natl Acad Sci USA.* (2014) 111:1–6. doi: 10.1073/pnas.1404671111
9. Asrir A, Aloulou M, Gador M, Pérals C, Fazilleau N. Interconnected subsets of memory follicular helper T cells have different effector functions. *Nat Commun.* (2017) 8:847. doi: 10.1038/s41467-017-00843-7
10. Fazilleau N, Eisenbraun MD, Malherbe L, Ebright JN, Pogue-Caley RR, McHeyzer-Williams LJ, et al. Lymphoid reservoirs of antigen-specific memory T helper cells. *Nat Immunol.* (2007) 8:753–61. doi: 10.1038/ni1472
11. Crotty S. A brief history of T cell help to B cells. *Nat Rev Immunol.* (2015) 15:185–9. doi: 10.1038/nri3803
12. Kawabe T, Naka T, Yoshida K, Tanaka T, Fujiwara H, Suematsu S, et al. The immune responses in CD40-deficient mice: impaired immunoglobulin class switching and germinal center formation. *Immunity* (1994) 1:167–78. doi: 10.1016/1074-7613(94)90095-7
13. Foy TM, Laman JD, Ledbetter JA, Aruffo A, Claassen E, Noelle RJ. gp39-CD40 interactions are essential for germinal center formation and the development of B cell memory. *J Exp Med.* (1994) 180:157–63. doi: 10.1084/jem.180.1.157
14. Takahashi Y, Dutta PR, Cerasoli DM, Kelsoe G. *In situ* studies of the primary immune response to (4-hydroxy-3-nitrophenyl)acetyl. V. Affinity maturation develops in two stages of clonal selection. *J Exp Med.* (1998) 187:885–95. doi: 10.1084/jem.187.6.885
15. Allen RC, Armitage RJ, Conley ME, Rosenblatt H, Jenkins NA, Copeland NG, et al. CD40 ligand gene defects responsible for X-linked hyper-IgM syndrome. *Science* (1993) 259:990–3. doi: 10.1126/science.7679801
16. Walker LSK, Gulbranson-Judge A, Flynn S, Brocker T, Raykundalia C, Goodall M, et al. Compromised OX40 function in CD28-deficient mice is linked with failure to develop CXCL13 chemokine receptor 5-positive CD4 cells and germinal centers. *J Exp Med.* (1999) 190:1115–22.
17. Ferguson SE, Han S, Kelsoe C, Thompson CB. CD28 is required for germinal center formation. *J Immunol.* (1996) 156:4576–81.
18. Borriello F, Sethna MP, Boyd SD, Schweitzer AN, Tivol EA, Jacoby D, et al. B7-1 and B7-2 have overlapping, critical roles in immunoglobulin class switching and germinal center formation. *Immunity* (1997) 6:303–13. doi: 10.1016/S1074-7613(00)80333-7
19. Haynes NM, Allen CDC, Lesley R, Ansel KM, Killeen N, Cyster JG. Role of CXCR5 and CCR7 in follicular Th cell positioning and appearance of a programmed cell death gene-1 high germinal center-associated subpopulation. *J Immunol.* (2007) 179:5099–108. doi: 10.4049/jimmunol.179.8.5099
20. Moriyama S, Takahashi N, Green JA, Hori S, Kubo M, Cyster JG, et al. Sphingosine-1-phosphate receptor 2 is critical for follicular helper T cell retention in germinal centers. *J Exp Med.* (2014) 211:1297–305. doi: 10.1084/jem.20131666
21. Wang CJ, Heuts F, Ovcinnikovs V, Wardzinski L, Bowers C, Schmidt EM, et al. CTLA-4 controls follicular helper T-cell differentiation by regulating the strength of CD28 engagement. *Proc Natl Acad Sci USA.* (2015) 112:524–9. doi: 10.1073/pnas.1414576112
22. Walker LSK, Sansom DM. Confusing signals: recent progress in CTLA-4 biology. *Trends Immunol.* (2015) 36:63–70. doi: 10.1016/j.it.2014.12.001
23. Walker LSK, Sansom DM. The emerging role of CTLA4 as a cell-extrinsic regulator of T cell responses. *Nat Rev Immunol.* (2011) 11:852–63. doi: 10.1038/nri3108
24. Wing JB, Ise W, Kurosaki T, Sakaguchi S. Regulatory T cells control antigen-specific expansion of Tfh cell number and humoral immune responses via the coreceptor CTLA-4. *Immunity* (2014) 41:1013–25. doi: 10.1016/j.immuni.2014.12.006
25. Sage PT, Paterson AM, Lovitch SB, Sharpe AH. The coinhibitory receptor CTLA-4 controls B cell responses by modulating T follicular helper, T follicular regulatory, and T regulatory cells. *Immunity* (2014) 41:1026–39. doi: 10.1016/j.immuni.2014.12.005
26. Linterman MA, Pierson W, Lee SK, Kallies A, Kawamoto S, Rayner TF, et al. Foxp3+ follicular regulatory T cells control the germinal center response. *Nat Med.* (2011) 17:975–82. doi: 10.1038/nm.2425
27. Wing JB, Tekgüç M, Sakaguchi S. Control of germinal center responses by T-follicular regulatory cells. *Front Immunol.* (2018) 9:1910. doi: 10.3389/fimmu.2018.01910
28. Fazilleau N, Aloulou M. Several follicular regulatory T cell subsets with distinct phenotype and function emerge during germinal center reactions. *Front Immunol.* (2018) 9:1792. doi: 10.3389/fimmu.2018.01792
29. Xie MM, Dent AL. Unexpected help: follicular regulatory T cells in the germinal center. *Front Immunol.* (2018) 9:1536. doi: 10.3389/fimmu.2018.01536
30. Flynn S, Toellner K-M, Raykundalia C, Goodall M, Lane P. CD4 T cell cytokine differentiation: the B cell activation molecule, OX40 ligand, instructs CD4 T cells to express interleukin 4 and upregulates expression of the chemokine receptor, Bcl-1. *J Exp Med.* (1998) 188:297–304. doi: 10.1084/jem.188.2.297
31. Brocker T, Gulbranson-Judge A, Flynn S, Riedinger M, Raykundalia C, Lane P. CD4 T cell traffic control: *in vivo* evidence that ligation of OX40 on CD4 T cells by OX40-ligand expressed on dendritic cells leads to the accumulation of CD4 T cells in B follicles. *Eur J Immunol.* (1999) 29:1610–6. doi: 10.1002/(SICI)1521-4141(199905)29:05<1610::AID-IMMU1610>3.0.CO;2-8
32. Boettler T, Moeckel F, Cheng Y, Heeg M, Salek-Ardakani S, Crotty S, et al. OX40 facilitates control of a persistent virus infection. *PLoS Pathog.* (2012) 8:e1002913. doi: 10.1371/journal.ppat.1002913
33. Tahilian V, Hutchinson TE, Abboud G, Croft M, Salek-Ardakani S. OX40 cooperates with ICOS to amplify follicular Th cell development and germinal center reactions during infection. *J Immunol.* (2017) 198:218–28. doi: 10.4049/jimmunol.1601356
34. Cortini A, Ellinghaus U, Malik TH, Cunningham-Graham DS, Botto M, Vyse TJ. B cell OX40L supports T follicular helper cell development and contributes to SLE pathogenesis. *Ann Rheum Dis.* (2017) 76:2095–103. doi: 10.1136/annrheumdis-2017-211499
35. Boettler T, Choi YS, Salek-Ardakani S, Cheng Y, Moeckel F, Croft M, et al. Exogenous OX40 stimulation during *Lymphocytic choriomeningitis virus* infection impairs follicular Th cell differentiation and diverts CD4 T cells into the effector lineage by upregulating blimp-1. *J Immunol.* (2013) 191:5026–35. doi: 10.4049/jimmunol.1300013
36. Johnston RJ, Poholek AC, DiToro D, Yusuf I, Eto D, Barnett B, et al. Bcl6 and Blimp-1 are reciprocal and antagonistic regulators of T follicular helper cell differentiation. *Science* (2009) 325:1006–10. doi: 10.1126/science.1175870
37. Marriott CL, Mackley EC, Ferreira C, Veldhoen M, Yagita H, Withers DR. OX40 controls effector CD4+ T-cell expansion, not follicular T helper cell generation in acute *Listeria* infection. *Eur J Immunol.* (2014) 44:2437–47. doi: 10.1002/eji.201344211
38. Akiba H, Takeda K, Kojima Y, Usui Y, Harada N, Yamazaki T, et al. The role of ICOS in the CXCR5+ follicular B helper T cell maintenance *in vivo*. *J Immunol.* (2005) 175:2340–8. doi: 10.4049/jimmunol.175.4.2340
39. McAdam AJ, Greenwald RJ, Levin MA, Chernova T, Malenkovich N, Ling V, et al. ICOS is critical for CD40-mediated antibody class switching. *Nature* (2001) 409:102–5. doi: 10.1038/35051107
40. Tafuri A, Shahinian A, Bladt F, Yoshinaga SK, Jordana M, Wakeham A, et al. ICOS is essential for effective T-helper-cell responses. *Nature* (2001) 409:105–9. doi: 10.1038/35051113
41. Dong C, Juedes AE, Temann UA, Shrestha S, Allison JP, Ruddle NH, et al. ICOS co-stimulatory receptor is essential for T-cell activation and function. *Nature* (2001) 409:97–101. doi: 10.1038/35051100

42. Dong C, Temann UA, Flavell RA. Cutting edge: critical role of inducible costimulator in germinal center reactions. *J Immunol.* (2001) 166:3659–62. doi: 10.4049/jimmunol.166.6.3659
43. Choi YS, Kageyama R, Eto D, Escobar TC, Johnston RJ, Monticelli L, et al. ICOS receptor instructs T follicular helper cell versus effector cell differentiation via induction of the transcriptional repressor Bcl6. *Immunity* (2011) 34:932–46. doi: 10.1016/j.immuni.2011.03.023
44. Weber JP, Fuhrmann F, Feist RK, Lahmann A, Al Baz MS, Gentz L-J, et al. ICOS maintains the T follicular helper cell phenotype by down-regulating Krüppel-like factor 2. *J Exp Med.* (2015) 212:217–33. doi: 10.1084/jem.20141432
45. Gigoux M, Shang J, Pak Y, Xu M, Choe J, Mak TW, et al. Inducible costimulator promotes helper T-cell differentiation through phosphoinositide 3-kinase. *Proc Natl Acad Sci USA.* (2009) 106:20371–6. doi: 10.1073/pnas.0911573106
46. Walker LSK, Wiggett HE, Gaspal FMC, Raykundalia CR, Goodall MD, Toellner K-MM, et al. Established T cell-driven germinal center B cell proliferation is independent of CD28 signaling but is tightly regulated through CTLA-4. *J Immunol.* (2003) 170:91–8. doi: 10.4049/jimmunol.170.1.91
47. Webb LMC, Linterman MA. Signals that drive T follicular helper cell formation. *Immunology* (2017) 152:185–94. doi: 10.1111/imm.12778
48. Linterman MA, Denton AE, Divekar DP, Zvetkova I, Kane L, Ferreira C, et al. CD28 expression is required after T cell priming for helper T cell responses and protective immunity to infection. *Elife* (2014) 3:1–21. doi: 10.7554/eLife.03180
49. Mak TW, Shahinian A, Yoshinaga SK, Wakeham A, Boucher LM, Pintlilie M, et al. Costimulation through the inducible costimulator ligand is essential for both T helper and B cell functions in T cell-dependent B cell responses. *Nat Immunol.* (2003) 4:765–72. doi: 10.1038/ni947
50. Nurieva RI, Chung Y, Hwang D, Yang XO, Kang HS, Ma L, et al. Generation of T Follicular helper cells is mediated by interleukin-21 but independent of T helper 1, 2, or 17 cell lineages. *Immunity* (2008) 29:138–49. doi: 10.1016/j.immuni.2008.05.009
51. Xu H, Li X, Liu D, Li J, Zhang X, Chen X, et al. Follicular T-helper cell recruitment governed by bystander B cells and ICOS-driven motility. *Nature* (2013) 496:523–7. doi: 10.1038/nature12058
52. Bossaller L, Burger J, Draeger R, Grimbacher B, Knoth R, Plebani A, et al. ICOS Deficiency is associated with a severe reduction of CXCR5+CD4 germinal center Th cells. *J Immunol.* (2006) 177:4927–32. doi: 10.4049/jimmunol.177.7.4927
53. Warnatz K, Bossaller L, Salzer U, Skrabl-Baumgartner A, Schwinger W, Van Der Burg M, et al. Human ICOS deficiency abrogates the germinal center reaction and provides a monogenic model for common variable immunodeficiency. *Blood* (2006) 107:3045–52. doi: 10.1182/blood-2005-07-2955
54. Allen CDC, Okada T, Tang HL, Cyster JG. Imaging of germinal center selection events during affinity maturation. *Science* (2007) 315:528–31. doi: 10.1126/science.1136736
55. Okada T, Miller MJ, Parker I, Krummel MF, Neighbors M, Hartley SB, et al. Antigen-engaged B cells undergo chemotaxis toward the T zone and form motile conjugates with helper T cells. *PLoS Biol.* (2005) 3:e150. doi: 10.1371/journal.pbio.0030150
56. Qi H, Cannons JL, Klauschen F, Schwartzberg PL, Germain RN. SAP-controlled T-B cell interactions underlie germinal centre formation. *Nature* (2008) 455:764–9. doi: 10.1038/nature07345
57. Cannons JL, Qi H, Lu KT, Dutta M, Gomez-Rodriguez J, Cheng J, et al. Optimal germinal center responses require a multistage T cell:B cell adhesion process involving integrins, SLAM-associated protein, and CD84. *Immunity* (2010) 32:253–65. doi: 10.1016/j.immuni.2010.01.010
58. Czar MJ, Kersh EN, Mijares LA, Lanier G, Lewis J, Yap G, et al. Altered lymphocyte responses and cytokine production in mice deficient in the X-linked lymphoproliferative disease gene SH2D1A/DSHP/SAP. *Proc Natl Acad Sci USA.* (2001) 98:7449–54. doi: 10.1073/pnas.131193098
59. Crotty S, Kersh EN, Cannons J, Schwartzberg PL, Ahmed R. SAP is required for generating long term humoral immunity. *Nature* (2003) 421:282–7. doi: 10.1038/nature01318
60. Cannons JL, Yu LJ, Jankovic D, Crotty S, Horai R, Kirby M, et al. SAP regulates T cell-mediated help for humoral immunity by a mechanism distinct from cytokine regulation. *J Exp Med.* (2006) 203:1551–65. doi: 10.1084/jem.20052097
61. Yusuf I, Kageyama R, Monticelli L, Johnston RJ, Ditoro D, Hansen K, et al. Germinal center T follicular helper cell IL-4 production is dependent on signaling lymphocytic activation molecule receptor (CD150). *J Immunol.* (2010) 185:190–202. doi: 10.4049/jimmunol.0903505
62. Cannons JL, Tangye SG, Schwartzberg PL. SLAM family receptors and SAP adaptors in immunity. *Annu Rev Immunol.* (2011) 29:665–705. doi: 10.1146/annurev-immunol-030409-101302
63. Ballesteros-Tato A, León B, Graf BA, Moquin A, Adams PS, Lund FE, et al. Interleukin-2 inhibits germinal center formation by limiting T follicular helper cell differentiation. *Immunity* (2012) 36:847–56. doi: 10.1016/j.immuni.2012.02.012
64. Johnston RJ, Choi YS, Diamond JA, Yang JA, Crotty S. STAT5 is a potent negative regulator of T FH cell differentiation. *J Exp Med.* (2012) 209:243–50. doi: 10.1084/jem.20111174
65. Li J, Lu E, Yi T, Cyster JG. EBI2 augments Tfh cell fate by promoting interaction with IL-2-quenching dendritic cells. *Nature* (2016) 533:110–4. doi: 10.1038/nature17947
66. Crotty S. Follicular Helper CD4 T Cells (T FH). *Annu Rev Immunol.* (2011) 29:621–63. doi: 10.1146/annurev-immunol-031210-101400
67. Ma CS, Suryani S, Avery DT, Chan A, Nanan R, Santner-Nanan B, et al. Early commitment of nave human CD4+ T cells to the T follicular helper (FH) cell lineage is induced by IL-12. *Immunol Cell Biol.* (2009) 87:590–600. doi: 10.1038/icc.2009.64
68. Schmitt N, Morita R, Bourdery L, Benteibibel SE, Zurawski SM, Banchereau J, et al. Human dendritic cells induce the differentiation of interleukin-21-producing T follicular helper-like cells through interleukin-12. *Immunity* (2009) 31:158–69. doi: 10.1016/j.immuni.2009.04.016
69. Shulman Z, Gitlin AD, Weinstein JS, Lainez B, Esplugues E, Flavell RA, et al. Dynamic signaling by T follicular helper cells during germinal center B cell selection. *Science* (2014) 345:1058–62. doi: 10.1126/science.1257861
70. Linterman MA, Rigby RJ, Wong RK, Yu D, Brink R, Cannons JL, et al. Follicular helper T cells are required for systemic autoimmunity. *J Exp Med.* (2009) 206:561–76. doi: 10.1084/jem.20081886
71. Silva DG, Daley SR, Hogan J, Lee SK, Teh CE, Hu DY, et al. Anti-islet autoantibodies trigger autoimmune diabetes in the presence of an increased frequency of islet-reactive CD4 T cells. *Diabetes* (2011) 60:2102–11. doi: 10.2337/db10-1344
72. Kenefick R, Wang CJ, Kapadi T, Wardzinski L, Attridge K, Clough LE, et al. Follicular helper T cell signature in type 1 diabetes. *J Clin Invest.* (2015) 125:292–303. doi: 10.1172/JCI76238
73. Chevalier N, Macia L, Tan JK, Mason LJ, Robert R, Thorburn AN, et al. The role of follicular helper T cell molecules and environmental influences in autoantibody production and progression to inflammatory arthritis in mice. *Arthritis Rheumatol.* (2016) 68:1026–38. doi: 10.1002/art.39481
74. Teng F, Klinger CN, Felix KM, Bradley CP, Wu E, Tran NL, et al. Gut Microbiota Drive autoimmune arthritis by promoting differentiation and migration of peyer's patch T follicular helper cells. *Immunity* (2016) 44:875–88. doi: 10.1016/j.immuni.2016.03.013
75. Moschovakis GL, Bubke A, Friedrichsen M, Falk CS, Feederle R, Förster R. T cell specific Cxcr5 deficiency prevents rheumatoid arthritis. *Sci Rep.* (2017) 7:1–13. doi: 10.1038/s41598-017-08935-6
76. Tangye SG, Ma CS, Brink R, Deenick EK. The good, the bad and the ugly — Tfh cells in human health and disease. *Nat Rev Immunol.* (2013) 13:412–26. doi: 10.1038/nri3447
77. Vinuesa CG, Linterman MA, Yu D, MacLennan ICM. Follicular helper T cells. *Annu Rev Immunol.* (2016) 34:335–68. doi: 10.1146/annurev-immunol-041015-055605
78. Ueno H. T follicular helper cells in human autoimmunity. *Curr Opin Immunol.* (2016) 43:24–31. doi: 10.1016/j.coi.2016.08.003
79. Shulman Z, Gitlin AD, Targ S, Jankovic M, Pasqual G, Nussenzweig MC, et al. T follicular helper cell dynamics in germinal centers. *Science* (2013) 341:673–7. doi: 10.1126/science.1241680

80. Lühje K, Kallies A, Shimohakamada Y, Belz GT, Light A, Tarlinton DM, et al. The development and fate of follicular helper T cells defined by an IL-21 reporter mouse. *Nat Immunol.* (2012) 13:491–8. doi: 10.1038/ni.2261
81. Liu X, Yan X, Zhong B, Nurieva RI, Wang A, Wang X, et al. Bcl6 expression specifies the T follicular helper cell program *in vivo*. *J Exp Med.* (2012) 209:1841–52. S1–24. doi: 10.1084/jem.20120219
82. Weber JP, Fuhrmann F, Hutloff A. T-follicular helper cells survive as long-term memory cells. *Eur J Immunol.* (2012) 42:1981–8. doi: 10.1002/eji.201242540
83. Choi YS, Yang JA, Yusuf I, Johnston RJ, Greenbaum J, Peters B, et al. Bcl6 expressing follicular helper CD4T cells are fate committed early and have the capacity to form memory. *J Immunol.* (2013) 190:4014–26. doi: 10.4049/jimmunol.1202963
84. Hale JS, Youngblood B, Latner DR, Mohammed AUR, Ye L, Akondy RS, et al. Distinct memory cd4+ t cells with commitment to t follicular helper- and t helper 1-cell lineages are generated after acute viral infection. *Immunity* (2013) 38:805–17. doi: 10.1016/j.immuni.2013.02.020
85. Kitano M, Moriyama S, Ando Y, Hikida M, Mori Y, Kurosaki T, et al. Bcl6 Protein expression shapes pre-germinal center B cell dynamics and follicular helper T cell heterogeneity. *Immunity* (2011) 34:961–72. doi: 10.1016/j.immuni.2011.03.025
86. Locci M, Havenar-Daughton C, Landais E, Wu J, Kroenke MA, Arleham CL, et al. Human circulating PD-1+CXCR3-CXCR5+ memory Tfh cells are highly functional and correlate with broadly neutralizing HIV antibody responses. *Immunity* (2013) 39:758–69. doi: 10.1016/j.immuni.2013.08.031
87. Morita R, Schmitt N, Bentebibel SE, Ranganathan R, Bourdery L, Zurawski G, et al. Human blood CXCR5+CD4+T cells are counterparts of t follicular cells and contain specific subsets that differentially support antibody secretion. *Immunity* (2011) 34:108–21. doi: 10.1016/j.immuni.2010.12.012
88. Chevalier N, Jarrossay D, Ho E, Avery DT, Ma CS, Yu D, et al. CXCR5 expressing human central memory CD4T cells and their relevance for humoral immune responses. *J Immunol.* (2011) 186:5556–68. doi: 10.4049/jimmunol.1002828
89. He J, Tsai LM, Leong YA, Hu X, Ma CS, Chevalier N, et al. Circulating precursor CCR7loPD-1hi CXCR5+ CD4+ T cells indicate Tfh cell activity and promote antibody responses upon antigen reexposure. *Immunity* (2013) 39:770–81. doi: 10.1016/j.immuni.2013.09.007
90. Boswell KL, Paris R, Boritz E, Ambrozak D, Yamamoto T, Darko S, et al. Loss of circulating CD4T cells with B cell helper function during chronic HIV infection. *PLoS Pathog.* (2014) 10:e1003853. doi: 10.1371/journal.ppat.1003853
91. Sage PT, Alvarez D, Godec J, Andrian UH von, Sharpe AH. Circulating T follicular regulatory and helper cells have memory-like properties. *J Clin Invest.* (2014) 124:5191–204. doi: 10.1172/JCI76861
92. Viisanen T, Ihantola E-L, Nääntö-Salonen K, Hyöty H, Nurminen N, Selvenius J, et al. Circulating CXCR5+PD-1+ICOS+ follicular T helper cells are increased close to the diagnosis of type 1 diabetes in children with multiple autoantibodies. *Diabetes* (2017) 66:437–447. doi: 10.2337/db16-0714
93. Serr I, Fürst RW, Ott VB, Scherm MG, Nikolaev A, Gökmen F, et al. miRNA92a targets KLF2 and the phosphatase PTEN signaling to promote human T follicular helper precursors in T1D islet autoimmunity. *Proc Natl Acad Sci USA.* (2016) 113:E6659–68. doi: 10.1073/pnas.1606646113
94. He J, Zhang X, Wei Y, Sun X, Chen Y, Deng J, et al. Low-dose interleukin-2 treatment selectively modulates CD4+ T cell subsets in patients with systemic lupus erythematosus. *Nat Med.* (2016) 22:991–3. doi: 10.1038/nm.4148
95. Fahey LM, Wilson EB, Elsaesser H, Fistonich CD, McGavern DB, Brooks DG. Viral persistence redirects CD4T cell differentiation toward T follicular helper cells. *J Exp Med.* (2011) 208:987–99. doi: 10.1084/jem.20101773
96. Fernando MMA, Stevens CR, Walsh EC, De Jager PL, Goyette P, Plenge RM, et al. Defining the role of the MHC in autoimmunity: a review and pooled analysis. *PLoS Genet* (2008) 4:e1000024. doi: 10.1371/journal.pgen.1000024
97. Ooi JD, Petersen J, Tan yu H, Huynh M, Willett ZJ, Ramarathinam SH, et al. Dominant protection from HLA-linked autoimmunity by antigen-specific regulatory T cells. *Nature* (2017) 545:243–7. doi: 10.1038/nature22329
98. MacArthur J, Bowler E, Cerezo M, Gil L, Hall P, Hastings E, et al. The new NHGRI-EBI Catalog of published genome-wide association studies (GWAS Catalog). *Nucleic Acids Res.* (2017) 45:D896–901. doi: 10.1093/nar/gkw1133
99. Gough SCL, Walker LSK, Sansom DM. CTLA4 gene polymorphism and autoimmunity. *Immunol Rev.* (2005) 204:102–15. doi: 10.1111/j.0105-2896.2005.00249.x
100. Ueda H, Howson JMM, Esposito L, Heward J, Snook H, Chamberlain G, et al. Association of the T-cell regulatory gene CTLA4 with susceptibility to autoimmune disease. *Nature* (2003) 423:506–11. doi: 10.1038/nature01621
101. Bluestone JA, St. Clair EW, Turka LA. CTLA4lg: bridging the basic immunology with clinical application. *Immunity* (2006) 24:233–8. doi: 10.1016/j.immuni.2006.03.001
102. Mittereder N, Kuta E, Bhat G, Da Costa K, Cheng LI, Herbst R, et al. Loss of immune tolerance is controlled by ICOS in Sle1 mice. *J Immunol.* (2016) 197:491–503. doi: 10.4049/jimmunol.1502241
103. Peters AL, Stunz LL, Bishop GA. CD40 and autoimmunity: the dark side of a great activator. *Semin Immunol.* (2009) 21:293–300. doi: 10.1016/j.smim.2009.05.012
104. Kong S, Thiruppathi M, Qiu Q, Lin Z, Dong H, Chini EN, et al. DBC1 is a suppressor of B cell activation by negatively regulating alternative NF- κ B transcriptional activity. *J Immunol.* (2014) 193:5515–24. doi: 10.4049/jimmunol.1401798
105. Webb GJ, Hirschfield GM, Lane PJJ. OX40, OX40L and autoimmunity: a comprehensive review. *Clin Rev Allergy Immunol.* (2016) 50:312–32. doi: 10.1007/s12016-015-8498-3
106. Pociot F, Akolkar B, Concannon P, Erlich HA, Julier C, Morahan G, et al. Genetics of type 1 diabetes: what's next? *Diabetes* (2010) 59:1561–71. doi: 10.2337/db10-0076
107. Parrish-Novak J, Dillon SR, Nelson A, Hammond A, Sprecher C, Gross JA, et al. Interleukin 21 and its receptor are involved in NK cell expansion and regulation of lymphocyte function. *Nature* (2000) 408:57–63. doi: 10.1038/35040504
108. Ozaki K, Kikly K, Michalovich D, Young PR, Leonard WJ. Cloning of a type I cytokine receptor most related to the IL-2 receptor beta chain. *Proc Natl Acad Sci USA.* (2000) 97:11439–44. doi: 10.1073/pnas.200360997
109. Weinstein JS, Herman EI, Lainez B, Licona-Limón P, Esplugues E, Flavell R, et al. TFH cells progressively differentiate to regulate the germinal center response. *Nat Immunol.* (2016) 17:1197–205. doi: 10.1038/ni.3554
110. Chevrier S, Kratina T, Emslie D, Tarlinton DM, Corcoran LM. IL4 and IL21 cooperate to induce the high Bcl6 protein level required for germinal center formation. *Immunol Cell Biol.* (2017) 95:925–32. doi: 10.1038/icb.2017.71
111. Wu Y-Y, Kumar R, Haque MS, Castillejo-Lopez C, Alarcon-Riquelme ME. BANK1 Controls CpG-Induced IL-6 Secretion via a p38 and MNK1/2/eIF4E translation initiation pathway. *J Immunol.* (2013) 191:6110–6. doi: 10.4049/jimmunol.1301203
112. Chavele K-M, Merry E, Ehrenstein MR. Cutting edge: circulating plasmablasts induce the differentiation of human T follicular helper cells via IL-6 production. *J Immunol.* (2015) 194:2482–5. doi: 10.4049/jimmunol.1401190
113. Maine CJ, Marquardt K, Cheung J, Sherman LA. PTPN22 controls the germinal center by influencing the numbers and activity of T follicular helper cells. *J Immunol.* (2014) 192:1415–24. doi: 10.4049/jimmunol.1302418
114. Wiede F, Sacirbegovic F, Leong YA, Yu D, Tiganis T. PTPN2-deficiency exacerbates T follicular helper cell and B cell responses and promotes the development of autoimmunity. *J Autoimmun.* (2016) 76:85–100. doi: 10.1016/j.jaut.2016.09.004
115. Allen CDCC, Ansel KM, Low C, Lesley R, Tamamura H, Fujii N, et al. Germinal center dark and light zone organization is mediated by CXCR4 and CXCR5. *Nat Immunol.* (2004) 5:943–52. doi: 10.1038/ni1100
116. Pereira J, Kelly L, Xu Y, Cyster J. EBV induced molecule-2 mediates B cell segregation between outer and center follicle. *Nature* (2009) 460:1122–6. doi: 10.1038/nature08226
117. Gatto D, Paus D, Basten A, Mackay CR, Brink R. Guidance of B cells by the orphan G protein-coupled receptor EBI2 shapes humoral immune responses. *Immunity* (2009) 31:259–69. doi: 10.1016/j.immuni.2009.06.016
118. Suan D, Nguyen A, Moran I, Bourne K, Hermes JR, Arshi M, et al. T follicular helper cells have distinct modes of migration and molecular signatures in naive and memory immune responses. *Immunity* (2015) 42:704–18. doi: 10.1016/j.immuni.2015.03.002
119. Kageyama R, Cannons JL, Zhao F, Yusuf I, Lao C, Locci M, et al. The receptor Ly108 functions as a SAP adaptor-dependent on-off switch for T

- cell help to B cells and NKT cell development. *Immunity* (2012) 36:986–1002. doi: 10.1016/j.immuni.2012.05.016
120. Ise W, Kohyama M, Schraml BU, Zhang T, Schwer B, Basu U, et al. The transcription factor BATF controls the global regulators of class-switch recombination in both B cells and T cells. *Nat Immunol.* (2011) 12:536–43. doi: 10.1038/ni.2037
121. Bollig N, Brüstle A, Kellner K, Ackermann W, Abass E, Raifer H, et al. Transcription factor {IRF4} determines germinal center formation through follicular T-helper cell differentiation. *Proc Natl Acad Sci USA.* (2012) 109:8664–9. doi: 10.1073/pnas.1205834109
122. Kroenke MA, Eto D, Locci M, Cho M, Davidson T, Haddad EK, et al. Bcl6 and Maf cooperate to instruct human follicular helper CD4 T cell differentiation. *J Immunol.* (2012) 188:3734–44. doi: 10.4049/jimmunol.1103246
123. Ochiai K, Maienschein-Cline N, Simonetti G, Chen J, Rosenthal R, Brink R, et al. Transcriptional regulation of germinal center B and plasma cell fates by dynamical control of IRF4. *Immunity* (2013) 38:918–29. doi: 10.1016/j.immuni.2013.04.009
124. Stauss D, Brunner C, Berberich-Siebelt F, Höpken UE, Lipp M, Müller G. The transcriptional coactivator Bob1 promotes the development of follicular T helper cells via Bcl6. *EMBO J.* (2016) 35:881–98. doi: 10.15252/embj.201591459
125. Chen G, Hardy K, Bunting K, Daley S, Ma L, Shannon MF. Regulation of the IL-21 gene by the NF- B transcription factor c-Rel. *J Immunol.* (2010) 185:2350–9. doi: 10.4049/jimmunol.1000317
126. Neyt K, Perros F, GeurtsvanKessel CH, Hammad H, Lambrecht BN. Tertiary lymphoid organs in infection and autoimmunity. *Trends Immunol.* (2012) 33:297–305. doi: 10.1016/j.it.2012.04.006
127. Corsiero E, Nerviani A, Bombardieri M, Pitzalis C. Ectopic lymphoid structures: powerhouse of autoimmunity. *Front Immunol.* (2016) 7:430. doi: 10.3389/fimmu.2016.00430
128. Rao DA, Gurish MF, Marshall JL, Slowikowski K, Fonseka CY, Liu Y, et al. Pathologically expanded peripheral T helper cell subset drives B cells in rheumatoid arthritis. *Nature* (2017) 542:110–4. doi: 10.1038/nature20810
129. Vu Van D, Beier KC, Pietzke LJ, Al Baz MS, Feist RK, Gurka S, et al. Local T/B cooperation in inflamed tissues is supported by T follicular helper-like cells. *Nat Commun.* (2016) 7:10875. doi: 10.1038/ncomms10875
130. Manzo A, Vitolo B, Humby F, Caporali R, Jarrossay D, Dell'Accio F, et al. Mature antigen-experienced T helper cells synthesize and secrete the B cell chemoattractant CXCL13 in the inflammatory environment of the rheumatoid joint. *Arthritis Rheum.* (2008) 58:377–87. doi: 10.1002/art.23966
131. Fabris M, de Vita S, Blasone N, Visentini D, Pezzarini E, Pontarini E, et al. Serum levels of anti-CCP antibodies, anti-MCV antibodies and RF iga in the follow-up of patients with rheumatoid arthritis treated with rituximab. *Autoimmun Highlights* (2010) 1:87–94. doi: 10.1007/s13317-010-0013-5
132. Cambridge G, Perry HC, Nogueira L, Serre G, Parsons HM, De La Torre I, et al. The effect of B-cell depletion therapy on serological evidence of B-cell and plasmablast activation in patients with rheumatoid arthritis over multiple cycles of rituximab treatment. *J Autoimmun.* (2014) 50:67–76. doi: 10.1016/j.jaut.2013.12.002
133. Yusuf I, Stern J, McCaughy TM, Gallagher S, Sun H, Gao C, et al. Germinal center B cell depletion diminishes CD4+ follicular T helper cells in autoimmune mice. *PLoS ONE* (2014) 9:e102791. doi: 10.1371/journal.pone.0102791
134. Xu X, Shi Y, Cai Y, Zhang Q, Yang F, Chen H, et al. Inhibition of increased circulating Tfh cell by anti-CD20 monoclonal antibody in patients with type 1 diabetes. *PLoS ONE* (2013) 8:e79858. doi: 10.1371/journal.pone.0079858
135. Verstappen GM, Kroese FGM, Meiners PM, Corneth OB, Huitema MG, Haacke EA, et al. B cell depletion therapy normalizes circulating follicular TH cells in primary Sjögren syndrome. *J Rheumatol.* (2017) 44:49–58. doi: 10.3899/jrheum.160313
136. Wallin EF, Jolly EC, Suchánek O, Bradley JA, Espéli M, Jayne DRW, et al. Human T-follicular helper and T-follicular regulatory cell maintenance is independent of germinal centers. *Blood* (2014) 124:2666–74. doi: 10.1182/blood-2014-07-585976
137. Kim CH, Rott LS, Clark-Lewis I, Campbell DJ, Wu L, Butcher EC. Subspecialization of CXCR5+ T cells: B helper activity is focused in a germinal center-localized subset of CXCR5+ T cells. *J Exp Med.* (2001) 193:1373–81. doi: 10.1084/jem.193.12.1373
138. Rasheed A-U, Rahn H-P, Sallusto F, Lipp M, Müller G. Follicular B helper T cell activity is confined to CXCR5hiICOShi CD4 T cells and is independent of CD57 expression. *Eur J Immunol.* (2006) 36:1892–903. doi: 10.1002/eji.200636136
139. Benteibibel S-E, Schmitt N, Banchereau J, Ueno H. Human tonsil B-cell lymphoma 6 (BCL6)-expressing CD4+ T-cell subset specialized for B-cell help outside germinal centers. *Proc Natl Acad Sci USA.* (2011) 108:E488–97. doi: 10.1073/pnas.1100898108
140. Montalban X, Hauser SL, Kappos L, Arnold DL, Bar-Or A, Comi G, et al. Ocrelizumab versus placebo in primary progressive multiple sclerosis. *N Engl J Med.* (2017) 376:209–20. doi: 10.1056/NEJMoa1606468
141. Hauser SL, Bar-Or A, Comi G, Giovannoni G, Hartung H-P, Hemmer B, et al. Ocrelizumab versus interferon beta-1a in relapsing multiple sclerosis. *N Engl J Med.* (2017) 376:221–34. doi: 10.1056/NEJMoa1601277
142. Alvarez E, Piccio L, Mikesell RJ, Trinkaus K, Parks BJ, Naismith RT, et al. Predicting optimal response to B-cell depletion with rituximab in multiple sclerosis using CXCL13 index, magnetic resonance imaging and clinical measures. *Mult Scler J Exp Transl Clin.* (2015) 1:205521731562380. doi: 10.1177/2055217315623800

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2018 Petersone, Edner, Ovcinnikovs, Heuts, Ross, Ntavli, Wang and Walker. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



Lymph Node Cellular Dynamics in Cancer and HIV: What Can We Learn for the Follicular CD4 (Tfh) Cells?

Antigoni Poultisidi¹, Yiannis Dimopoulos², Ting-Fang He³, Triantafyllos Chavakis⁴, Emmanouil Saloustros⁵, Peter P. Lee^{3*} and Constantinos Petrovas^{2*}

¹ Department of Surgery, Medical School, University of Thessaly, Larissa, Greece, ² Tissue Analysis Core, Immunology Laboratory, Vaccine Research Center, NIAID, NIH, Bethesda, MD, United States, ³ Department of Immuno-Oncology, Beckman Research Institute, City of Hope Comprehensive Cancer Center, Duarte, CA, United States, ⁴ Institute of Clinical Chemistry and Laboratory Medicine, Technische Universität Dresden, Dresden, Germany, ⁵ Department of Internal Medicine, Medical School, University of Thessaly, Larissa, Greece

OPEN ACCESS

Edited by:

Georgia Fousteri,
San Raffaele Hospital (IRCCS), Italy

Reviewed by:

Guido Ferrari,
Duke University, United States
Karin Schillbach,
Universität Tübingen, Germany

*Correspondence:

Peter P. Lee
plee@coh.org
Constantinos Petrovas
petrovasc@mail.nih.gov

Specialty section:

This article was submitted to
T Cell Biology,
a section of the journal
Frontiers in Immunology

Received: 10 May 2018

Accepted: 07 September 2018

Published: 27 September 2018

Citation:

Poultisidi A, Dimopoulos Y, He T-F, Chavakis T, Saloustros E, Lee PP and Petrovas C (2018) Lymph Node Cellular Dynamics in Cancer and HIV: What Can We Learn for the Follicular CD4 (Tfh) Cells?.
Front. Immunol. 9:2233.
doi: 10.3389/fimmu.2018.02233

Lymph nodes (LNs) are central in the generation of adaptive immune responses. Follicular helper CD4 T (Tfh) cells, a highly differentiated CD4 population, provide critical help for the development of antigen-specific B cell responses within the germinal center. Throughout the past decade, numerous studies have revealed the important role of Tfh cells in Human Immunodeficiency Virus (HIV) pathogenesis as well as in the development of neutralizing antibodies post-infection and post-vaccination. It has also been established that tumors influence various immune cell subsets not only in their proximity, but also in draining lymph nodes. The role of local or tumor associated lymph node Tfh cells in disease progression is emerging. Comparative studies of Tfh cells in chronic infections and cancer could therefore provide novel information with regards to their differentiation plasticity and to the mechanisms regulating their development.

Keywords: cancer, HIV, Tfh cells, lymph nodes, follicles

INTRODUCTION

Given the important role the lymphatic system has in combating foreign pathogens, changes in Lymph Node (LN) architecture/cellularity have been recognized in a variety of infectious diseases. In Human Immunodeficiency Virus (HIV) infection, LNs play a central role for disease pathogenesis. Early studies revealed the covert infection of CD4 T cells in the LN and its role in the depletion of these cells throughout disease progress (1, 2). From a prognostic point of view, the degree of follicular structure damage has been used for classification of disease progress (3, 4). Current vaccine strategies targeting the humoral arm of the immune system have revealed the need for a comprehensive understanding of follicular dynamics. Given the role of T follicular helper (Tfh) cells as an HIV reservoir (5) and as critical “helpers” in the development of antibodies (6), understanding their biology is of great interest.

Besides infectious diseases, LNs are involved in various forms of neoplasia, either as metastasis or as primary disease sites (i.e., lymphomas). It is of critical importance to distinguish between infectious etiologies of lymphadenopathy vs. neoplastic causes (7). In non-hematopoietic neoplastic disease, LNs are involved in disease progression (a) as part of the regional disease, contributing to local morbid phenomena when infiltrated by the tumor, (b) as metastatic disease *per se* (N status in the Tumor Node Metastasis staging system)—affecting the treatment/management of patients, and (c) as a mediator for propagating further distant metastasis. Although LN

involvement has major prognostic implications for the patient and is thus incorporated in the staging strategies of neoplasms (8), the clinical management of the lymphatic system draining a malignancy is an area of ongoing research and trends are shifting accordingly. For example, in breast cancer patients attention has been drawn to the Sentinel Lymph Node (SLN) (9), which is defined as the first LN or group of LNs that interstitial fluid and cells from the tumor microenvironment pass through on their route to the venous circulation via lymphatic vessels (10–12). Despite the different etiology and specific pathways/molecular factors operating selectively in cancer or infectious diseases, the comparative analysis of LN immunodynamics could provide important information regarding the development and maintenance of Tfh cells.

LYMPH NODES: ORGANIZATION, TFH CELLS

LN provide the site of initiation of adaptive immune responses and are strategically placed along lymphatic vessels (13). Antigen presenting cells (APCs) initiate immune responses via interactions with T and B cells that gain access to specific regions of the LN (14). Functionally, the LNs can be separated into lobules (13). The structural backbone of the lobule is comprised of Fibroblastic Reticular Cells (FRCs) and their fibers, which support the parenchyma, provide routes for the migration of lymphocytes, and facilitate the interaction between lymphocytes and APCs (4). The inter-follicular regions of the cortex and the paracortex are mainly populated by T cells, which gain access to the parenchyma by migrating through high endothelial venules (HEVs), following the CCR7/CCL19, CCL21 axis (4). These cells interact with dendritic cells (DCs) that have reached the LN via the afferent lymphatic vessels and HEVs (4). Primary follicles (located in the cortex) contain mainly naïve B cells, whereas secondary follicles are recognized by the formation of a germinal center (GC) (13). GCs, the antibody production factory of the body, are populated by antigen stimulated B cells, follicular dendritic cells (FDCs), Tfh cells, and macrophages- among other cell types (15, 16). FDCs can present antigens and stimulatory signals to GC B and T cells, as well as produce CXCL-13, the ligand for CXCR5 (17), while tingible body macrophages are capable of phagocytizing dying cells (13).

Tfh cells provide critical signals for the activation, isotype switching, affinity maturation, and differentiation of B cells into memory B cells and plasma cells via surface bound receptors (i.e., PD-1, ICOS, CD40) (18, 19) and secreted factors like IL-21 and IL-4 (20, 21), that support the GC responses by regulating the differentiation of both Tfh and B cells through the activation of STAT signaling pathways (21–25). The spatial organization of Tfh cells is regulated, at least, by (i) chemokine gradients (i.e., CXCL-13 and CXCL-10/IP-10, a chemokine produced by macrophages and acting on CXCR3) enabling their trafficking toward GC (17, 26). and (ii) function of signaling pathways mediating their retention within the follicular/GC areas. GC homing is accomplished via downregulation of CCR7 and upregulation of CXCR5- a process mediated by Bcl-6, a critical

transcription factor for Tfh cell differentiation (27, 28). Once inside the GC, S1PR1 family receptors aid in Tfh cell retention in GCs- this is accomplished by downregulation of S1PR1 and upregulation of S1PR2 (29–31). Dynamic positioning inside the GC is influenced by the local production of factors, one of which is CXCL-12/SDF-1 that acts on CXCR4 (32, 33). The unique PD-1^{hi}CXCR5^{hi} phenotype has been widely used for the identification of Tfh cells (34, 35). In line with this, imaging studies have shown the highly skewed localization of PD-1^{hi} CD4 T cells within the GC areas (GC-Tfh cells) (35–37). High expression per cell (judged by Mean Fluorescence Intensity-MFI) of other surface receptors (like ICOS and TIGIT), is selectively found on the vast majority of GC-Tfh cells (35, 37, 38). The differential expression of surface receptors like CD150 and CD57 can further delineate GC-Tfh subpopulations (35, 37, 39). For example, Tfh cells expressing lower levels of CD150 (SLAM) secrete higher levels of IL-4 and are thought to be more differentiated than Tfh cells expressing higher levels of CD150 (37, 39). The presence of PD-1^{dim}CXCR5^{hi} (non-GC) Tfh (34), the differential expression of CXCR3 (Th1-like Tfh cells) (40, 41), or Tfh master regulators like Bcl-6 (5, 28, 35), further adds to the heterogeneity of the Tfh pool. We should emphasize, though, that different follicular CD4 T cell subsets are presumably exposed to different local signals within the follicle. Delineation of these signals, as well as the connection between phenotype and function of Tfh cell subsets, is an important step toward the comprehensive understanding of Tfh cell biology and their role in human diseases.

A separate group of CD4 T cells located in the follicle/GC- and particularly the T-B area border (42)- are T follicular regulatory (Tfr) cells (43), which possibly originate from thymic T regulatory (Treg) cells, after adaption of their gene expression profile to include -apart from FoxP3- factors and receptors expressed in Tfh cells, such as Bcl-6 and CXCR5 (38, 44, 45). A mutual regulation between Tfh and GC B cells through the function of receptor/ligand axes (such as CD40/CD40L, ICOS-ICOSL) has been proposed (46–48). In a similar manner, Tfr cells can suppress GC reactivity (43) either by altering such mutual regulation- a process mediated in part by CTLA-4 (49)- or by directly affecting B or Tfh cells (43, 50). Although they represent a small minority of follicular CD4 T cells, the presence of Tfr cells aids in limiting the GC response to prevent uncontrolled B cell proliferation and the consequences thereof, such as the production of antibodies that recognize “self” antigens (37, 44).

LYMPH NODES IN NEOPLASMS

The Concept of SLNs

From an immunological perspective, SLNs are the site where tumor antigen loaded APCs encounter naïve T and B cells, leading to the generation of immune responses against neoplasms (51–53). A relatively smaller distance from the primary tumor site presumably increases the possibility for the SLN to be affected by the tumor than downstream draining LNs (DLNs) are, potentially leading to the variable immune responses observed in DLNs depending on the distance from the primary tumor (54). Factors which modulate these responses may tip the balance

from control to tolerance/spread of the neoplasm (51–53). Most research has focused on SLNs in the context of melanoma and breast cancer patients, but knowledge is expanding about SLNs in other types of neoplasms—such as genitourinary, pulmonary, and gastrointestinal tumors (11). However, accurate identification of SLNs can be very challenging (55). Rerouting of lymphatic flow or the presence of tumor cells in the subcapsular sinus may affect the ability to detect the SLN with the help of dyes (55, 56). Lymphangiogenesis and alterations of lymph flow dynamics induced by the tumor may also alter lymphatic drainage (55). Therefore, a false negative SLN (up to 9.8% in breast cancer) could result in under-staging and mistreating patients (57, 58).

Structural Alterations of SLNs Induced by Tumors

Tumors can affect structural components in the SLNs and DLNs even before metastasis to these sites has occurred, creating an environment fostering tumor cell invasion to the SLN (59–61). Major structural SLN changes have been described, related to (a) increased lymphatic drainage from the tumor to the LNs (55), which can induce biophysical remodeling/changes of the LN matrix (62) and potentially lead to the activation of signaling pathways [i.e., transforming growth factor (TGF)- β pathway] associated with tumor spreading/induction of immune suppression in the LNs, in a similar fashion to what has been observed in primary tumor sites (63), (b) increased lymphangiogenesis and angiogenesis induced by vascular endothelial growth factors (VEGFs) originating from the tumor environment, which can ultimately contribute to the spreading of tumor cells to the SLN and beyond (55, 60, 64–66), and (c) a “flatter” morphology of the endothelial cells of the HEVs, which can potentially lead to impaired access of naïve T cells to the LN parenchyma (53). Overall, these structural changes in the lymphatics and the vasculature can set the stage for future metastasis of cancer cells to SLNs (Figure 1).

Non-follicular Immune Dynamics in SLNs

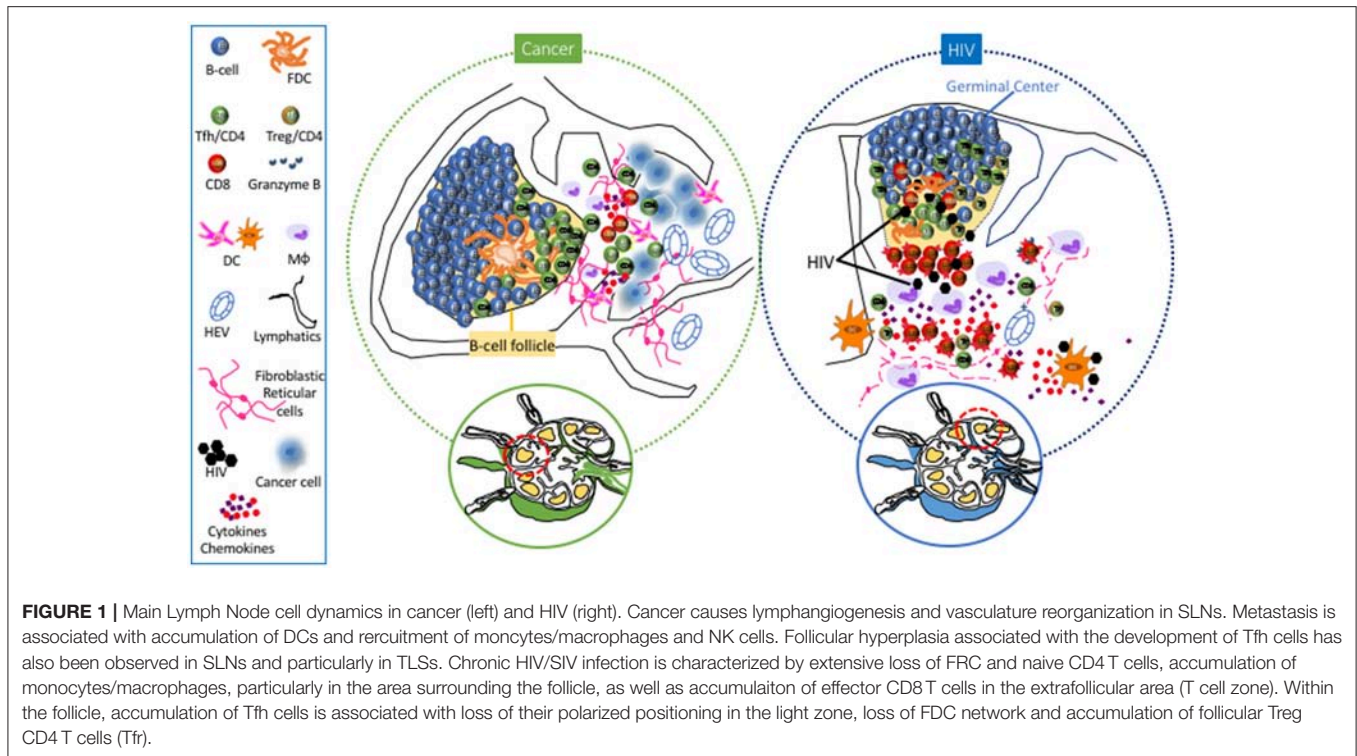
Apart from structural changes, modulations of immune cell subsets have also been observed to precede actual metastasis of tumor cells to SLNs. Several studies suggest a compromised capacity to induce a “favorable” Th-1 response against the tumor due to (i) decreased DC density and clustering in paracortical LN regions (67–70) and (ii) compromised DC function (60, 70–72). Conversely, other studies have advocated increased presence of activated DCs in SLNs, prior to the appearance of metastatic cells (73–75). However, transition to a mature DC phenotype and Th-1 cytokine response was noted after metastasis of breast tumor cells to the SLN, possibly reflecting antigenic stimulation against these cells (72) (Figure 1). Emerging studies have investigated the role of LN NK cells (76) and monocytes/macrophages in anti-tumor immunity (77, 78) and their targeting for adjuvant immunotherapies that could improve treatment of patients with metastatic cancer. Regarding adaptive immunity, alterations in cell types with prognostic implications (68) have been observed in SLNs and DLNs. Reduced numbers of CD4 and CD8 T cells (60, 64, 68) with an immunosuppressed profile (79) was found in SLNs, a profile associated with accumulation of FoxP3+

Treg CD4 cells in LNs harboring metastases (80–83) and worse prognosis/more widespread nodal disease in melanoma, breast, and gastric cancer (82–85). Various cytokines (GM-CSF, IL-2) are being investigated as a way to reverse this immune suppression and assist in the immune system’s effort to combat the neoplastic cells (53).

Follicular Dynamics in SLNs and Tertiary Lymphoid Structures (TLSs)

In contrast to extrafollicular cell dynamics, much less is known about follicular/B cell dynamics in the context of neoplastic disease. In SLNs, B cells—via the secretion of VEGF-A—could induce lymphangiogenesis and angiogenesis (61, 86, 87), potentially promoting the spread of tumors via lymphatics (55). However, an extended lymphatic network can lead to increased recruitment of DCs from the periphery to the LN (86), which could ultimately benefit the development of anti-tumor adaptive immunity. A trend toward improved 5-year survival was noted in melanoma patients, whose SLNs demonstrated follicular hyperplasia/GC accumulation (88). Besides their prognostic value for disease-free survival in breast cancer patients (89), SLN B cells is the source of affinity matured B cell clones that produced anti-tumor immunoglobulins detected in the blood (90). Investigation of such antibodies could potentially lead to the recognition of tumor antigens recognized by the immune system, which can subsequently be targeted in the context of immunotherapies (90). Furthermore, these findings imply that development of tumor-specific Tfh cells could be a critical factor for an effector response to a tumor. The progressive differentiation of Tfh cells within the follicular area, associated with differential localization and an orchestrated production of IL-21 and IL-4, provide critical signals for the isotype switching and differentiation of GC B cells by modulating transcription factors like Bcl-6 and Blimp-1 (21, 24, 91). In a mouse tumor model, accumulation of Tfh cells was noted in DLNs, along with a concomitant increase in IL-4 produced by these cells (92). On the other hand, recent studies have shown a beneficial role of IL-21 in cancer immunotherapy strategies (93, 94), possibly by modulating CD8 T cell response (95–97). Therefore, Tfh cells could potentially support antitumor immunity in ways extending past the help they provide to B cells.

The role of B cell infiltration in primary tumors is not clear (98, 99), with studies showing both a negative (100, 101) or a positive (102–105) effect on the antitumor immune responses. However, B cells contribute to the formation of tertiary lymphoid structures (TLSs)—defined as accumulations of lymphocytes in proximity to the primary tumor—which are associated with better prognosis (99) (Figure 1). Similar to B cells, an increased presence of Tfh cells in the primary tumor site has been associated with better clinical outcomes in breast (106) and non-small cell lung carcinoma (107). High expression of molecules like CXCL-13 and IL-21 (106, 108) by TLS associated Tfh cells contributes to the formation/organization of TLSs in the primary breast tumor and potentially contribute to the immune systems’ reaction (109). Presumably, TLSs facilitate the *in situ* production and secretion of anti-tumor antibodies that



could represent a mechanism to maximize the efficiency of adaptive immunity against tumors (99). A variety of regulatory cell subsets have the ability to influence Tfh cell function. In breast cancer, LN Treg cells can promote malignancy through a TGF β -1 mediated upregulation of the oncogenic receptor IL17rb (110). A coevolution of Treg cells and CXCL-13^{hi} Tfh cells in the TLSs was found, with the ratio between these two populations being a critical factor for tumor control by benefiting the development of an anti-tumor humoral response (109). Furthermore, the presence of myeloid-derived suppressor cells within the LN could potentially be a negative regulator for Tfh cells (111, 112), adding to the complexity of the regulation of these cells. Characterization of relevant cytokine producers and their spatial positioning within anatomically separated LN areas would be highly informative in understanding their potential role in regulating Tfh cell dynamics in SLN and TLSs.

Several reports have been focused on the characterization of circulating CXCR5^{hi} CD4 T (cTfh) cell subsets as a counterpart of the LN bona fide Tfh cells (113, 114). The lineage origin of cTfh cells and their direct association to LN Tfh cells is not clear (115, 116). Lower cTfh cells in the blood of hepatocellular carcinoma patients were associated with worse prognosis (117), while a higher frequency of “Th-1” CXCR3^{hi} cTfh cells was negatively associated with survival in gastric cancer (118). In breast cancer, a higher frequency of “exhausted” Tim-3^{hi} cTfh cells associated with higher expression of PD-1 per cell base was found - interestingly, *in vitro* blocking of Tim-3 increased the production of IL-21 and CXCL-13 by peripheral blood mononuclear cells (119). Future investigation of cTfhs in cancers of different

etiology could provide important information regarding their use as a biomarker, as well as their relationship to LN or TLS Tfh cells.

FOLLICULAR IMMUNE DYNAMICS: LESSONS FROM HIV/SIV (SIMIAN IMMUNODEFICIENCY VIRUS)

Structural Alterations

HIV infection leads to dramatic and progressive changes of LN architecture, especially evident during the chronic phase of infection (4). In reality, the degree of tissue damage has been used for the staging of disease (120). A major contributor to this damage is the extensive deposition of collagen (fibrosis) in the extrafollicular area (121), a process facilitated by increased levels of secreted TGF- β 1 from accumulated Treg cells (122, 123) and the activation of spatially associated fibroblasts (124, 125). Fibrosis leads to a vicious circle of naïve T cell pool and FRC network depletion (126, 127)- a network that provides the scaffold for cell migration (128) and vital signals for the recruitment (CCR7) (129, 130) and survival (IL-7) (130, 131) of naïve T cells (Figure 1). LN damage is associated with the persistent immune activation and tissue inflammation found in HIV/SIV (4). Despite the partial normalization of immunological parameters- such as CD4 counts, immune activation, and suppressed viremia- LN structure abnormalities persist in combination antiretroviral therapy (cART)-treated individuals (132–134), presumably affecting the development and function of LN relevant T cells -such as Tfh cells- in the context of new infections or vaccination (36).

Non-follicular Immune Dynamics

Besides tissue architecture, HIV/SIV infection has a major impact on the cellular dynamics within the extrafollicular areas. Monocytes/macrophages that express low levels of CD4 and other HIV coreceptors (135) can contribute to HIV/SIV pathogenesis by (i) supporting the viral reservoir, particularly in advanced disease or immunocompromised states (136, 137), and (ii) secreting inflammatory mediators like IL-6 and IL-10 (138), which play an important role in the development of GC responses (139). The accumulation of monocytic-lineage and plasmacytoid dendritic cells (pDCs) in LNs during acute SIV infection (140–143) is followed by their impaired function (leading to decreased production of cytokines like IFN- α and IL-12, which *in vitro* support T cell proliferation) during the chronic phase of infection (144–146). Despite the loss of both pDCs and myeloid DCs (mDCs) from lymphoid tissues and blood in chronic infection, LN-derived mDCs retain their functionality, especially the induction of Treg cells— an important regulator of Tfh cell function and GC reactivity (147, 148). Chronic HIV/SIV is characterized by the relative loss of LN CD4 cells— mainly attributed to loss of naïve CD4 T cells (39, 126, 149)— accompanied by an increased frequency of effector CD8 T cells (149) (**Figure 1**). Besides the direct killing of infected CD4 T cells, the cellular and molecular mechanisms regulating the LN T cell dynamics in HIV/SIV are not well understood. Structure damage, immune activation, inflammatory signals, and altered tissue chemokine gradients could all play an important role in this process. Recent studies have shown that chronic HIV/SIV infection is associated with sequestration of monocytes/macrophages around the follicular areas (150). Their possible role in LN CD8 T cell dynamics is supported by their (i) correlation with LN CD8 T cell in chronic SIV (149), (ii) spatial proximity to accumulated CD8 T cells in LN areas (149, 150), and (iii) potential to change local chemokine gradients through the secretion of chemokines like CXCL-9 and CXCL-10 (IP10) (151, 152), ligands for the CXCR3 receptor that is broadly expressed on LN CD8 T cells (149). Such altered chemokine gradients could contribute to LN T cell dynamics by modulating their (i) recruitment from the circulation (153, 154) and (ii) intra lymph node trafficking (26).

Follicular Dynamics

Understanding the follicular/GC— and particularly Tfh cell— immune dynamics in HIV/Simian Immunodeficiency Virus (SIV) infection is of great importance for (i) the identification of molecules/pathways associated with the development of broadly neutralizing antibodies that could inform the design of novel vaccine strategies targeting relevant GC cell populations and (ii) understanding the establishment and maintenance of a major viral reservoir (5), even in cART treated donors (155). To this end, the non-human primate (NHP) SIV model has provide invaluable information regarding the Tfh cell dynamics during infection. A relatively delayed development of Tfh cells during acute SIV has been described in peripheral LNs (39). Interestingly, different kinetics between spleen and LN associated Tfh cells has been found, indicating a differential regulation of Tfh cells in different lymphoid organs (156, 157). Chronic

HIV/SIV infection is associated with an altered (a) frequency (39), (b) function and signaling (39, 156), (c) molecular profile (39, 158), and (d) localization/distribution within the follicular areas (159) of Tfh cells (**Figure 1**). The dynamics of LN Tfh cells— associated with follicular hyperplasia in the LNs and hypergammaglobulinemia in the plasma (39, 160–162)— have been linked to progression to AIDS (162, 163), as well as to immune activation and associated cytokines— such as IL-6 and IFN- γ (19, 39, 161). The dependence of Tfh cells on immune activation and tissue inflammation is further supported by their downregulation in cART individuals (5, 160) and by the lack of accumulation of Tfh cells in LNs from infected African Green Monkeys (AGMs) (149), a natural host of the virus with no signs of immune activation (164). Apart from the altered frequency, SIV infection has a significant impact on the molecular signature of Tfh cells— characterized by upregulation of IFN- γ and TGF- β related genes (39)— indicating an increased response of Tfh cells to relevant stimuli and a role of TGF- β as regulator of Tfh cell dysfunction in chronic infection. The combination of (i) an increased expression of CXCL-13 in Tfh cells (39), (ii) a favorable phosphorylation of STAT3 (a positive regulator of Tfh cells (39, 158)) over STAT1 (39), and (iii) an increased expression of the IL-6/IL-6R axis found in chronic SIV (39) provides a molecular basis for the accumulation of Tfh cells in chronic HIV/SIV. Besides Bcl-6, SIV infection induces the expression of c-Maf (157), a master regulator of Tfh cells (139). Interestingly, a higher expression of T-bet (a Th-1 regulator) was found selectively in LN Tfh cells (157), in line with the accumulation of Th1-like Tfh cells in chronic SIV (165). The relative presence of such Tfh populations could have a significant effect on HIV/SIV pathogenesis (165).

Despite the accumulation of Tfh cells (5, 39) and GC B cells (39, 156), the majority of HIV-infected individuals do not develop broadly neutralizing antibodies against HIV (166), pointing to a perturbation of the Tfh-B cell interaction within the GC (167). Increased frequencies of Gag-specific compared to Env-specific Tfh cells found in chronic HIV (5, 39) could reflect a preferential development of Gag-specific Tfh cells or increased turnover of Env-specific Tfh cells. Application of cutting-edge technologies like single cell deep sequencing would be highly informative to this end. Analysis of Simian-Human immunodeficiency virus (SHIV) infected NHPs revealed that besides the frequency, the quality (judged by the expression of IL-21 vs. IFN- γ) of Env-specific Tfh responses was strongly associated with the development of broadly neutralizing antibodies in those animals (168). The increased expression of IL-21 found in HIV-specific Tfh cells (5, 160), could be counterbalanced by a reduced expression of IL-4 by Tfh cells (39), indicating that the development of broadly neutralizing analysis requires the orchestrated expression and activity of relevant cells and soluble mediators.

Other mechanisms that could contribute to the impaired functionality of Tfh cells in chronic HIV/SIV include (i) the high expression of PD-L1 on germinal center B cells, interacting with the highly expressed PD-1 on Tfh cells (52), (ii) the relative accumulation of potential suppressor Tfr cells (169) and (iii) the presence of follicular regulatory CD8 T cells (170). Besides

the frequency and quality of relevant cells, preservation of the follicular structure is a critical determinant for the development of GC responses in HIV infection- which is characterized by the loss of the Follicular Dendritic Cells (FDC) network and factors secreted by this network, such as CXCL-13 (36). Recent imaging studies revealed that preservation of FDC was associated with maintenance of Tfh cells and preservation of their function in HIV infection, manifested by the response of infected individuals to vaccination (36) and possibly with their distribution within the GC (Figure 2).

HIV/SIV infection affects the dynamics of other LN cells, including CD8 T cells. An increased frequency of follicular CD8 T cells (149, 150, 165) - even within intact follicles (149)- has been observed, implying that infection counteracts local “firewalls” that naturally keep CD8 T cells outside the follicular area. Although the naturally induced HIV-specific cytotoxic CD8 lymphocytes are relatively excluded from the GC area (171), the increased overall presence of follicular CD8 T cells provides an opportunity for novel CD8-based immunotherapies, i.e., the use of bispecific antibodies to redirect these cells to kill HIV-infected cells (149, 172). Immune activation and tissue inflammation are important factors for the dynamics of both follicular CD4 and CD8 T cells in chronic infection (149). The excessive immune activation, however, possibly leads to a generalized, non-cognate driven expansion of these cell populations. One could hypothesize that these dynamics could potentially affect the function of virus-specific Tfh responses, i.e., through the aberrant production of Tfh-cytokines.

CONCLUDING REMARKS, FUTURE DIRECTIONS

Comparative studies using Tfh cells from diseases with different etiologies represent one way to better understand the molecular and cellular basis for their generation and maintenance. Specifically, such studies between HIV and cancer could inform for:

1. *The mechanisms of Tfh development/maintenance in the settings of a chronic disease.* Besides the relative frequency and spatial positioning, HIV/SIV infection changes the molecular

profile of Tfh cells too (39). Is this profile of chronically *in vivo* stimulated Tfh cells unique for HIV/SIV or there is a core, preserved molecular signature during Tfh cell development under different stimuli like cancer neoantigens? Does the etiology/type of cancer have an impact on this profile? Relevant studies will provide critical information about the plasticity of the Tfh cell differentiation program.

2. *The role of tissue inflammation in the development of human Tfh cell responses.* Despite the expected differences between HIV and cancer-including, among others, the (a) nature of antigenic stimulation (viral proteins compared to neoantigens), (b) magnitude/type of tissue architectural changes, (c) presence of virus within the follicle and its ability to infect Tfh cells *per se* in HIV infection-inflammation plays an important role in the pathogenesis of both diseases (172–174). Comparative investigation of LNs from HIV infected individuals and cancer patients could inform for the presence, spatial distribution and possible role of specific pro-inflammatory cellular/molecular mediators for the recruitment of T cell to LNs and their trafficking between LN compartments. General, non-cognate driven activation of LN CD4 T cells could differ between cancer and a chronic viral infection like HIV, with a presumably differential impact in the generation of Tfh cell responses. Therefore, the development of methodologies allowing for the detection of antigen-specific Tfh cells, especially at tissue level, is of great importance.
3. *The impact of tissue structure alterations on Tfh cell dynamics and the and local interplay between adaptive immunity and Tfh cells.* Tissue changes like vasculature reorganization and extracellular matrix organization are less studied in HIV compared to cancer LNs. On the other hand, common tissue structure alterations like fibrosis could contribute to Tfh cell dynamics by affecting the dynamics of innate and adaptive immune cells in the extrafollicular areas (167). HIV/SIV is characterized by the accumulation of LN and particularly follicular CD8 T cells (148, 149). The regulation of these dynamics as well as the role of fCD8 T cells in GC B cell responses is not well understood. Conversely, the role of cytokines, like IL-21 produced by Tfh cells too, as regulators of LN CD8 T cells is not clear either. Understanding the interplay between LN CD8 T cells and Tfh cells is of

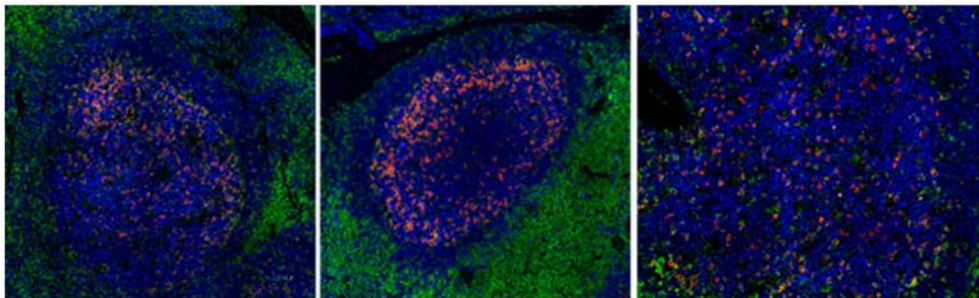


FIGURE 2 | Chronic HIV infection is associated with disturbed follicular organization. Reactive follicles from a healthy axillary (left), a breast cancer SLN (middle) and a HIV viremic axillary LN (right). The CD20 (blue), CD4 (green), and PD-1 (red) markers are shown.

great importance for LN immune responses both in HIV and cancer, particularly within TLSs, where sequestration of effector CD8 T cells in proximity to the tumor site could have an impact on disease progression.

4. *The “anatomical compartmentalization” of Tfh cell responses.* Are all Tfh cells across the human body the same? Recent studies have shown a differential regulation of Tfh cells between LNs and spleen in SIV infection (156). Is this the case for LN Tfh cells from different anatomical sites too? How that could affect the role of adaptive immunity responses developed in LN in the progression of cancer with different topology?
5. *The assessment of human Tfh cell quality.* Comparison of Tfh cells between HIV donors with broadly neutralizing antibodies and cancer donors with anti-tumor B cell response correlating with good prognosis could help define the spatial organization, the functional characteristics, and molecular signature of an “effective” Tfh cell response.

Given the limited, if any, access to LN tissues, especially from different time points throughout the course of disease, the discovery of circulating biomarkers recapitulating the germinal center reactivity is of great importance. Investigation of molecules like CXCL13 (37, 173) or cTfh cells (113) represents one direction in the hunt for biomarkers i.e., for monitoring the efficacy of vaccination protocols (175). Are cancers of different etiology associated with a particular phenotype/subset of cTfh cells? How do these cells compare to cTfh subsets found in HIV?

An in depth understanding of the Tfh cell development is a prerequisite for the designing of novel *in vivo* interventions aimed at boosting their function and developing effective B cell responses, particularly in HIV. Many questions are still open; how the prime/boost vaccination scheme affects the quality and breath of immunogen-specific Tfh responses? Combining optimal structure-based designed immunogens with new generation adjuvants or interventions targeting specific molecules/pathways involved in the generation of high quality Tfh cells could lead to more efficient vaccine strategies. On the other hand, any therapeutic intervention has to take in

consideration the best short and long-term oncological outcome, as well as survivors’ quality of life. Surgical interventions may be associated with morbidity/mortality. For example, axillary LN dissection in the context of breast cancer might result in an increased incidence of lifetime lymphedema (up to 21%), decreased arm mobility, and arm paresthesia (174). However, recent clinical trials are challenging the need for axillary surgery for tumors up to 2 cm (175, 176). New therapeutic schemes are urgently needed. The success of cancer immunotherapy in melanoma, lung cancer, renal cell carcinoma and other solid tumors has placed the power of T cell immunity into the armamentarium of cancer therapeutics. Similar immune therapies for patients with breast cancer are beginning to come to fruition, with most promising the use of PD-1 (e.g., pembrolizumab) and PD-L1 (e.g., avelumab) monoclonal antibodies (177, 178). However, the toxicity and cost of immunotherapies has revealed the need for predictive biomarkers. Potential biomarkers should be evaluated in the primary tumor, in the metastases, or in circulation. Given their role in breast cancer, the search for novel biomarkers at a LN and follicular level could be of great support for the decision of relevant surgical procedures.

AUTHOR CONTRIBUTIONS

AP, YD, TF-H, and ES wrote the manuscript. TC, PL, and CP conceived the idea and participated in the writing and editing the manuscript.

ACKNOWLEDGMENTS

Authors would like to thank the personnel of Tissue Analysis Core at VRC, NIAID for helpful discussions and suggestions and Dr E. Moysi for help with the cartoon figure. This research was supported by the Intramural Research Program of the Vaccine Research Center, NIAID, National Institutes of Health and CAVD grant (#OP1032325) from the Bill and Melinda Gates Foundation.

REFERENCES

1. Pantaleo G, Graziosi C, Demarest JE, Butini L, Montroni M, Fox CH, et al. HIV infection is active and progressive in lymphoid tissue during the clinically latent stage of disease. *Nature* (1993) 362:355–8. doi: 10.1038/362355a0
2. Embretson J, Zupancic M, Ribas JL, Burke A, Racz P, Tenner-Racz K, et al. Massive covert infection of helper T lymphocytes and macrophages by HIV during the incubation period of AIDS. *Nature* (1993) 362:359–62. doi: 10.1038/362359a0
3. Baroni CD, Uccini S. Lymph nodes in HIV-positive drug abusers with persistent generalized lymphadenopathy: histology, immunohistochemistry, and pathogenetic correlations. *Prog AIDS Pathol.* (1990) 2:33–50.
4. Estes JD. Pathobiology of HIV/SIV-associated changes in secondary lymphoid tissues. *Immunol Rev.* (2013) 254:65–77. doi: 10.1111/immr.12070
5. Perreau M, Savoye AL, De Crignis E, Corpataux JM, Cubas R, Haddad EK, et al. Follicular helper T cells serve as the major CD4 T cell compartment for HIV-1 infection, replication, and production. *J Exp Med.* (2013) 210:143–56. doi: 10.1084/jem.20121932
6. Breitfeld D, Ohl L, Kremmer E, Ellwart J, Sallusto F, Lipp M, et al. Follicular B helper T cells express CXC chemokine receptor 5, localize to B cell follicles, and support immunoglobulin production. *J Exp Med.* (2000) 192:1545–52. doi: 10.1084/jem.192.11.1545
7. Slack GW. The pathology of reactive lymphadenopathies: a discussion of common reactive patterns and their malignant mimics. *Arch Pathol Lab Med.* (2016) 140:881–92. doi: 10.5858/arpa.2015-0482-SA
8. Edge SB, Compton CC. The American Joint Committee on Cancer: the 7th edition of the AJCC cancer staging manual and the future of TNM. *Ann Surg Oncol.* (2010) 17:1471–4. doi: 10.1245/s10434-010-0985-4
9. Giuliano AE, Kirgan DM, Guenther JM, Morton DL. Lymphatic mapping and sentinel lymphadenectomy for breast cancer. *Ann Surg.* (1994) 220:391–8; discussion 8–401. doi: 10.1097/0000658-199409000-00015
10. Krag DN, Weaver DL. Pathological and molecular assessment of sentinel lymph nodes in solid tumors. *Semin Oncol.* (2002) 29:274–9. doi: 10.1053/sonc.2002.32894

11. Cochran AJ, Roberts AA, Saidat T. The place of lymphatic mapping and sentinel node biopsy in oncology. *Int J Clin Oncol.* (2003) 8:139–50. doi: 10.1007/s10147-003-0333-9
12. Uren RF, Thompson JF, Howman-Giles R. Sentinel lymph node biopsy in patients with melanoma and breast cancer. *Intern Med J.* (2001) 31:547–53. doi: 10.1046/j.1445-5994.2001.01282.x
13. Willard-Mack CL. Normal structure, function, and histology of lymph nodes. *Toxicol Pathol.* (2006) 34:409–24. doi: 10.1080/01926230600867727
14. Zeng M, Haase AT, Schacker TW. Lymphoid tissue structure and HIV-1 infection: life or death for T cells. *Trends Immunol.* (2012) 33:306–14. doi: 10.1016/j.it.2012.04.002
15. Mesin L, Ersching J, Vitoria GD. Germinal center B cell dynamics. *Immunity* (2016) 45:471–82. doi: 10.1016/j.immuni.2016.09.001
16. Vitoria GD, Schwickert TA, Fooksman DR, Kamphorst AO, Meyer-Hermann M, Dustin ML, et al. Germinal center dynamics revealed by multiphoton microscopy with a photoactivatable fluorescent reporter. *Cell* (2010) 143:592–605. doi: 10.1016/j.cell.2010.10.032
17. Ansel KM, Ngo VN, Hyman PL, Luther SA, Forster R, Sedgwick JD, et al. A chemokine-driven positive feedback loop organizes lymphoid follicles. *Nature* (2000) 406:309–14. doi: 10.1038/35018581
18. Crotty S. T follicular helper cell differentiation, function, and roles in disease. *Immunity* (2014) 41:529–42. doi: 10.1016/j.immuni.2014.10.004
19. Kim CH, Lim HW, Kim JR, Rott L, Hillsamer P, Butcher EC. Unique gene expression program of human germinal center T helper cells. *Blood* (2004) 104:1952–60. doi: 10.1182/blood-2004-03-1206
20. Shulman Z, Gitlin AD, Weinstein JS, Lainez B, Esplugues E, Flavell RA, et al. Dynamic signaling by T follicular helper cells during germinal center B cell selection. *Science* (2014) 345:1058–62. doi: 10.1126/science.1257861
21. Weinstein JS, Herman EI, Lainez B, Licona-Limon P, Esplugues E, Flavell R, et al. TFH cells progressively differentiate to regulate the germinal center response. *Nat Immunol.* (2016) 17:1197–205. doi: 10.1038/ni.3554
22. Wu Y, van Besouw NM, Shi Y, Hoogduijn MJ, Wang L, Baan CC. The biological effects of IL-21 signaling on B-cell-mediated responses in organ transplantation. *Front Immunol.* (2016) 7:319. doi: 10.3389/fimmu.2016.00319
23. Nurieva RI, Chung Y, Hwang D, Yang XO, Kang HS, Ma L, et al. Generation of T follicular helper cells is mediated by interleukin-21 but independent of T helper 1, 2, or 17 cell lineages. *Immunity* (2008) 29:138–49. doi: 10.1016/j.immuni.2008.05.009
24. Chevrier S, Kratina T, Emslie D, Tarlinton DM, Corcoran LM. IL4 and IL21 cooperate to induce the high Bcl6 protein level required for germinal center formation. *Immunity* (2017) 95:925–32. doi: 10.1038/icb.2017.71
25. Berglund LJ, Avery DT, Ma CS, Moens L, Deenick EK, Bustamante J, et al. IL-21 signalling via STAT3 primes human naive B cells to respond to IL-2 to enhance their differentiation into plasmablasts. *Blood* (2013) 122:3940–50. doi: 10.1182/blood-2013-06-506865
26. Groom JR, Richmond J, Murooka TT, Sorensen EW, Sung JH, Bankert K, et al. CXCR3 chemokine receptor-ligand interactions in the lymph node optimize CD4+ T helper 1 cell differentiation. *Immunity* (2012) 37:1091–103. doi: 10.1016/j.immuni.2012.08.016
27. Ballesteros-Tato A, Randall TD. Priming of T follicular helper cells by dendritic cells. *Immunity* (2014) 92:22–7. doi: 10.1038/icb.2013.62
28. Kroenke MA, Eto D, Locci M, Cho M, Davidson T, Haddad EK, et al. Bcl6 and Maf cooperate to instruct human follicular helper CD4 T cell differentiation. *J Immunol.* (2012) 188:3734–44. doi: 10.4049/jimmunol.1103246
29. Bankovich AJ, Shioh LR, Cyster JG. CD69 suppresses sphingosine 1-phosphate receptor-1 (S1P1) function through interaction with membrane helix 4. *J Biol Chem.* (2010) 285:22328–37. doi: 10.1074/jbc.M110.123299
30. Moriyama S, Takahashi N, Green JA, Hori S, Kubo M, Cyster JG, et al. Sphingosine-1-phosphate receptor 2 is critical for follicular helper T cell retention in germinal centers. *J Exp Med.* (2014) 211:1297–305. doi: 10.1084/jem.20131666
31. Blankenbach KV, Schwalm S, Pfeilschifter J, Meyer Zu Heringdorf D. Sphingosine-1-phosphate receptor-2 antagonists: therapeutic potential and potential risks. *Front Pharmacol.* (2016) 7:167. doi: 10.3389/fphar.2016.00167
32. Estes JD, Thacker TC, Hampton DL, Kell SA, Keele BF, Palenske EA, et al. Follicular dendritic cell regulation of CXCR4-mediated germinal center CD4 T cell migration. *J Immunol.* (2004) 173:6169–78. doi: 10.4049/jimmunol.173.10.6169
33. Elsner RA, Ernst DN, Baumgarth N. Single and coexpression of CXCR4 and CXCR5 identifies CD4 T helper cells in distinct lymph node niches during influenza virus infection. *J Virol.* (2012) 86:7146–57. doi: 10.1128/JVI.06904-11
34. Kohler SL, Pham MN, Folkvord JM, Arends T, Miller SM, Miles B, et al. Germinal center T follicular helper cells are highly permissive to HIV-1 and alter their phenotype during virus replication. *J Immunol.* (2016) 196:2711–22. doi: 10.4049/jimmunol.1502174
35. Alshekaili J, Chand R, Lee CE, Corley S, Kwong K, Papa I, et al. STAT3 regulates cytotoxicity of human CD57+ CD4+ T cells in blood and lymphoid follicles. *Sci Rep.* (2018) 8:3529. doi: 10.1038/s41598-018-21389-8
36. Moysi E, Pallikkuth S, De Armas LR, Gonzalez LE, Ambrozak D, George V, et al. Altered immune cell follicular dynamics in HIV infection following influenza vaccination. *J Clin Invest.* (2018) 128:3171–85. doi: 10.1172/JCI99884
37. Amodio D, Cotugno N, Macchiarulo G, Rocca S, Dimopoulos Y, Castrucci MR, et al. Quantitative multiplexed imaging analysis reveals a strong association between immunogen-specific B cell responses and tonsillar germinal center immune dynamics in children after influenza vaccination. *J Immunol.* (2018) 200:538–50. doi: 10.4049/jimmunol.1701312
38. Wu H, Chen Y, Liu H, Xu LL, Teuscher P, Wang S, et al. Follicular regulatory T cells repress cytokine production by follicular helper T cells and optimize IgG responses in mice. *Eur J Immunol.* (2016) 46:1152–61. doi: 10.1002/eji.201546094
39. Petrovas C, Yamamoto T, Gerner MY, Boswell KL, Wloka K, Smith EC, et al. CD4 T follicular helper cell dynamics during SIV infection. *J Clin Invest.* (2012) 122:3281–94. doi: 10.1172/JCI63039
40. Benteibibel SE, Khurana S, Schmitt N, Kurup P, Mueller C, Obermoser G, et al. ICOS(+)PD-1(+)CXCR3(+) T follicular helper cells contribute to the generation of high-avidity antibodies following influenza vaccination. *Sci Rep.* (2016) 6:26494. doi: 10.1038/srep26494
41. Velu V, Mylvaganam GH, Gangadhara S, Hong JJ, Iyer SS, Gumber S, et al. Induction of Th1-biased T follicular helper (Tfh) cells in lymphoid tissues during chronic simian immunodeficiency virus infection defines functionally distinct germinal center Tfh cells. *J Immunol.* (2016) 197:1832–42. doi: 10.4049/jimmunol.1600143
42. Sayin I, Radtke AJ, Vella LA, Jin W, Wherry EJ, Buggert M, et al. Spatial distribution and function of T follicular regulatory cells in human lymph nodes. *J Exp Med.* (2018) 215:1531–42. doi: 10.1084/jem.20171940
43. Linterman MA, Pierson W, Lee SK, Kallies A, Kawamoto S, Rayner TF, et al. Foxp3+ follicular regulatory T cells control the germinal center response. *Nat Med.* (2011) 17:975–82. doi: 10.1038/nm.2425
44. Vaeth M, Muller G, Stauss D, Dietz L, Klein-Hessling S, Serfling E, et al. Follicular regulatory T cells control humoral autoimmunity via NFAT2-regulated CXCR5 expression. *J Exp Med.* (2014) 211:545–61. doi: 10.1084/jem.20130604
45. Chung Y, Tanaka S, Chu F, Nurieva RI, Martinez GJ, Rawal S, et al. Follicular regulatory T cells expressing Foxp3 and Bcl-6 suppress germinal center reactions. *Nat Med.* (2011) 17:983–8. doi: 10.1038/nm.2426
46. Weinstein JS, Bertino SA, Hernandez SG, Poholek AC, Teplitzky TB, Nowyhed HN, et al. B cells in T follicular helper cell development and function: separable roles in delivery of ICOS ligand and antigen. *J Immunol.* (2014) 192:3166–79. doi: 10.4049/jimmunol.1302617
47. Baumjohann D, Preite S, Reboldi A, Ronchi F, Ansel KM, Lanzavecchia A, et al. Persistent antigen and germinal center B cells sustain T follicular helper cell responses and phenotype. *Immunity* (2013) 38:596–605. doi: 10.1016/j.immuni.2012.11.020
48. Liu D, Xu H, Shih C, Wan Z, Ma X, Ma W, et al. T-B-cell entanglement and ICOSL-driven feed-forward regulation of germinal centre reaction. *Nature* (2015) 517:214–8. doi: 10.1038/nature13803
49. Sage PT, Paterson AM, Lovitch SB, Sharpe AH. The coinhibitory receptor CTLA-4 controls B cell responses by modulating T follicular helper, T follicular regulatory, and T regulatory cells. *Immunity* (2014) 41:1026–39. doi: 10.1016/j.immuni.2014.12.005

50. Sage PT, Ron-Harel N, Juneja VR, Sen DR, Maleri S, Sunghak W, et al. Suppression by TFR cells leads to durable and selective inhibition of B cell effector function. *Nat Immunol.* (2016) 17:1436–46. doi: 10.1038/ni.3578
51. Kim R, Emi M, Tanabe K, Arihiro K. Immunobiology of the sentinel lymph node and its potential role for antitumor immunity. *Lancet Oncol.* (2006) 7:1006–16. doi: 10.1016/S1470-2045(06)70975-5
52. Takeuchi H, Kitajima M, Kitagawa Y. Sentinel lymph node as a target of molecular diagnosis of lymphatic micrometastasis and local immunoresponse to malignant cells. *Cancer Sci.* (2008) 99:441–50. doi: 10.1111/j.1349-7006.2007.00672.x
53. Cochran AJ, Huang RR, Lee J, Itakura E, Leong SP, Essner R. Tumour-induced immune modulation of sentinel lymph nodes. *Nat Rev Immunol.* (2006) 6:659–70. doi: 10.1038/nri1919
54. Cochran AJ, Pihl E, Wen DR, Hoon DS, Korn EL. Zoned immune suppression of lymph nodes draining malignant melanoma: histologic and immunohistologic studies. *J Natl Cancer Inst.* (1987) 78:399–405.
55. Karaman S, Detmar M. Mechanisms of lymphatic metastasis. *J Clin Invest.* (2014) 124:922–8. doi: 10.1172/JCI1606
56. Clough KB, Nasr R, Nos C, Vieira M, Ingenau C, Poulet B. New anatomical classification of the axilla with implications for sentinel node biopsy. *Br J Surg.* (2010) 97:1659–65. doi: 10.1002/bjs.7217
57. Krag DN, Anderson SJ, Julian TB, Brown AM, Harlow SP, Costantino JP, et al. Sentinel-lymph-node resection compared with conventional axillary-lymph-node dissection in clinically node-negative patients with breast cancer: overall survival findings from the NSABP B-32 randomised phase 3 trial. *Lancet Oncol.* (2010) 11:927–33. doi: 10.1016/S1470-2045(10)70207-2
58. Krag DN, Anderson SJ, Julian TB, Brown AM, Harlow SP, Ashikaga T, et al. Technical outcomes of sentinel-lymph-node resection and conventional axillary-lymph-node dissection in patients with clinically node-negative breast cancer: results from the NSABP B-32 randomised phase III trial. *Lancet Oncol.* (2007) 8:881–8. doi: 10.1016/S1470-2045(07)70278-4
59. Zuckerman NS, Yu H, Simons DL, Bhattacharya N, Carcamo-Cavazos V, Yan N, et al. Altered local and systemic immune profiles underlie lymph node metastasis in breast cancer patients. *Int J Cancer* (2013) 132:2537–47. doi: 10.1002/ijc.27933
60. Mansfield AS, Holtan SG, Grotz TE, Allred JB, Jakub JW, Erickson LA, et al. Regional immunity in melanoma: immunosuppressive changes precede nodal metastasis. *Mod Pathol.* (2011) 24:487–94. doi: 10.1038/modpathol.2010.227
61. Harrell MI, Iritani BM, Ruddell A. Tumor-induced sentinel lymph node lymphangiogenesis and increased lymph flow precede melanoma metastasis. *Am J Pathol.* (2007) 170:774–86. doi: 10.2353/ajpath.2007.060761
62. Rohner NA, McClain J, Tuell SL, Warner A, Smith B, Yun Y, et al. Lymph node biophysical remodeling is associated with melanoma lymphatic drainage. *FASEB J.* (2015) 29:4512–22. doi: 10.1096/fj.15-274761
63. Swartz MA, Lund AW. Lymphatic and interstitial flow in the tumour microenvironment: linking mechanobiology with immunity. *Nat Rev Cancer* (2012) 12:210–9. doi: 10.1038/nrc3186
64. Grotz TE, Jakub JW, Mansfield AS, Goldenstein R, Enninga EA, Nevala WK, et al. Evidence of Th2 polarization of the sentinel lymph node (SLN) in melanoma. *Oncoimmunology* (2015) 4:e1026504. doi: 10.1080/2162402X.2015.1026504
65. Balsat C, Blacher S, Herfs M, Van de Velde M, Signolle N, Sauthier P, et al. A specific immune and lymphatic profile characterizes the pre-metastatic state of the sentinel lymph node in patients with early cervical cancer. *Oncoimmunology* (2017) 6:e1265718. doi: 10.1080/2162402X.2016.1265718
66. Hirakawa S, Brown LF, Kodama S, Paavonen K, Alitalo K, Detmar M. VEGF-C-induced lymphangiogenesis in sentinel lymph nodes promotes tumor metastasis to distant sites. *Blood* (2007) 109:1010–7. doi: 10.1182/blood-2006-05-021758
67. van den Hout M, Koster BD, Sluijter BJR, Molenkamp BG, van de Ven R, van den Eertwegh AJM, et al. Melanoma sequentially suppresses different DC subsets in the sentinel lymph node, affecting disease spread and recurrence. *Cancer Immunol Res.* (2017) 5:969–77. doi: 10.1158/2326-6066.CIR-17-0110
68. Kohrt HE, Nouri N, Nowels K, Johnson D, Holmes S, Lee PP. Profile of immune cells in axillary lymph nodes predicts disease-free survival in breast cancer. *PLoS Med.* (2005) 2:e284. doi: 10.1371/journal.pmed.0020284
69. Cochran AJ, Morton DL, Stern S, Lana AM, Essner R, Wen DR. Sentinel lymph nodes show profound downregulation of antigen-presenting cells of the paracortex: implications for tumor biology and treatment. *Mod Pathol.* (2001) 14:604–8. doi: 10.1038/modpathol.3880358
70. Chang AY, Bhattacharya N, Mu J, Setiadi AF, Carcamo-Cavazos V, Lee GH, et al. Spatial organization of dendritic cells within tumor draining lymph nodes impacts clinical outcome in breast cancer patients. *J Transl Med.* (2013) 11:242. doi: 10.1186/1479-5876-11-242
71. Kara PP, Ayhan A, Caner B, Gultekin M, Ugur O, Bozkurt MF, et al. Analysis of dendritic cells in sentinel lymph nodes of patients with endometrial and patients with cervical cancers. *Int J Gynecol Cancer* (2009) 19:1239–43. doi: 10.1111/IGC.0b013e3181b3e616
72. Matsuura K, Yamaguchi Y, Ueno H, Osaki A, Arihiro K, Toge T. Maturation of dendritic cells and T-cell responses in sentinel lymph nodes from patients with breast carcinoma. *Cancer* (2006) 106:1227–36. doi: 10.1002/cncr.21729
73. Poindexter NJ, Sahin A, Hunt KK, Grimm EA. Analysis of dendritic cells in tumor-free and tumor-containing sentinel lymph nodes from patients with breast cancer. *Breast Cancer Res.* (2004) 6:R408–15. doi: 10.1186/bcr808
74. Sakakura K, Chikamatsu K, Sakurai T, Takahashi K, Murata T, Oriuchi N, et al. Infiltration of dendritic cells and NK cells into the sentinel lymph node in oral cavity cancer. *Oral Oncol.* (2005) 41:89–96. doi: 10.1016/j.oraloncology.2004.07.004
75. Bembek A, Li J, Lodenkemper C, Kemmner W, Stein H, Wernecke KD, et al. Presence of mature DC-Lamp+ dendritic cells in sentinel and non-sentinel lymph nodes of breast cancer patients. *Eur J Surg Oncol.* (2008) 34:514–8. doi: 10.1016/j.ejso.2007.05.013
76. Messaoudene M, Fregni G, Fourmentraux-Neves E, Chanal J, Maubec E, Mazouz-Dorval S, et al. Mature cytotoxic CD56(bright)/CD16(+) natural killer cells can infiltrate lymph nodes adjacent to metastatic melanoma. *Cancer Res.* (2014) 74:81–92. doi: 10.1158/0008-5472.CAN-13-1303
77. Shiota T, Miyasato Y, Ohnishi K, Yamamoto-Ibusuki M, Yamamoto Y, Iwase H, et al. The clinical significance of CD169-positive lymph node macrophage in patients with breast cancer. *PLoS ONE* (2016) 11:e0166680. doi: 10.1371/journal.pone.0166680
78. Cella M, Jarrossay D, Facchetti F, Aleardi O, Nakajima H, Lanzavecchia A, et al. Plasmacytoid monocytes migrate to inflamed lymph nodes and produce large amounts of type I interferon. *Nat Med.* (1999) 5:919–23. doi: 10.1038/11360
79. Schule JM, Bergkvist L, Hakansson L, Gustafsson B, Hakansson A. CD28 expression in sentinel node biopsies from breast cancer patients in comparison with CD3-zeta chain expression. *J Transl Med.* (2004) 2:45. doi: 10.1186/1479-5876-2-45
80. Viguier M, Lemaitre F, Verola O, Cho MS, Gorochov G, Dubertret L, et al. Foxp3 expressing CD4+CD25(high) regulatory T cells are overrepresented in human metastatic melanoma lymph nodes and inhibit the function of infiltrating T cells. *J Immunol.* (2004) 173:1444–53. doi: 10.4049/jimmunol.173.2.1444
81. Martin F, Ladoire S, Mignot G, Apetoh L, Ghiringhelli F. Human FOXP3 and cancer. *Oncogene* (2010) 29:4121–9. doi: 10.1038/ncr.2010.174
82. Mohos A, Sebestyen T, Liszkay G, Plotar V, Horvath S, Gaudi I, et al. Immune cell profile of sentinel lymph nodes in patients with malignant melanoma - FOXP3+ cell density in cases with positive sentinel node status is associated with unfavorable clinical outcome. *J Transl Med.* (2013) 11:43. doi: 10.1186/1479-5876-11-43
83. Mansfield AS, Heikkila PS, Vaara AT, von Smitten KA, Vakkila JM, Leidenius MH. Simultaneous Foxp3 and IDO expression is associated with sentinel lymph node metastases in breast cancer. *BMC Cancer* (2009) 9:231. doi: 10.1186/1471-2407-9-231
84. Nakamura R, Sakakibara M, Nagashima T, Sangai T, Arai M, Fujimori T, et al. Accumulation of regulatory T cells in sentinel lymph nodes is a prognostic predictor in patients with node-negative breast cancer. *Eur J Cancer* (2009) 45:2123–31. doi: 10.1016/j.ejca.2009.03.024
85. Lee HE, Park DJ, Kim WH, Kim HH, Lee HS. High FOXP3+ regulatory T-cell density in the sentinel lymph node is associated with downstream non-sentinel lymph-node metastasis in gastric cancer. *Br J Cancer* (2011) 105:413–9. doi: 10.1038/bjc.2011.248
86. Angeli V, Ginhoux F, Llodra J, Quemeneur L, Frenette PS, Skobe M, et al. B cell-driven lymphangiogenesis in inflamed lymph nodes

- enhances dendritic cell mobilization. *Immunity* (2006) 24:203–15. doi: 10.1016/j.immuni.2006.01.003
87. Ruddell A, Mezquita P, Brandvold KA, Farr A, Iritani BM. B lymphocyte-specific c-Myc expression stimulates early and functional expansion of the vasculature and lymphatics during lymphomagenesis. *Am J Pathol.* (2003) 163:2233–45. doi: 10.1016/S0002-9440(10)63581-X
88. Abbott J, Buckley M, Taylor LA, Xu G, Karakousis G, Czerniecki BJ, et al. Histological immune response patterns in sentinel lymph nodes involved by metastatic melanoma and prognostic significance. *J Cutan Pathol.* (2018) 45:377–86. doi: 10.1111/cup.13127
89. Blenman KR, He T-F, Frankel PH, Ruel NH, Schwartz EJ, Krag DN, et al. Sentinel lymph node B cells can predict disease-free survival in breast cancer patients. *npj Breast Cancer.* (2018) 4:28. doi: 10.1038/s41523-018-0081-7
90. McDaniel JR, Pero SC, Voss WN, Shukla GS, Sun Y, Schaetzle S, et al. Identification of tumor-reactive B cells and systemic IgG in breast cancer based on clonal frequency in the sentinel lymph node. *Cancer Immunol Immunother.* (2018) 67:729–38. doi: 10.1007/s00262-018-2123-2
91. Belanger S, Crotty S. Dances with cytokines, featuring TFH cells, IL-21, IL-4 and B cells. *Nat Immunol.* (2016) 17:1135–6. doi: 10.1038/ni.3561
92. Shirota H, Klinman DM, Ito SE, Ito H, Kubo M, Ishioka C. IL4 from T follicular helper cells downregulates antitumor immunity. *Cancer Immunol Res.* (2017) 5:61–71. doi: 10.1158/2326-6066.CIR-16-0113
93. Lewis KE, Selby MJ, Masters G, Valle J, Dito G, Curtis WR, et al. Interleukin-21 combined with PD-1 or CTLA-4 blockade enhances antitumor immunity in mouse tumor models. *Oncoimmunology* (2017) 7:e1377873. doi: 10.1080/2162402X.2017.1377873
94. Chen C, Liu X, Ren Y. Interleukin 21 treatment in a murine model as a novel potential cytokine immunotherapy for colon cancer. *Adv Clin Exp Med.* (2018) 27:583–9. doi: 10.17219/acem/68703
95. Xin G, Schauder DM, Lainez B, Weinstein JS, Dai Z, Chen Y, et al. A Critical role of IL-21-Induced BATF in sustaining CD8-T-cell-mediated chronic viral control. *Cell Rep.* (2015) 13:1118–24. doi: 10.1016/j.celrep.2015.09.069
96. Frohlich A, Kisielow J, Schmitz I, Freigang S, Shamsheev AT, Weber J, et al. IL-21R on T cells is critical for sustained functionality and control of chronic viral infection. *Science* (2009) 324:1576–80. doi: 10.1126/science.1172815
97. Raeber ME, Zurbuchen Y, Impellizzeri D, Boyman O. The role of cytokines in T-cell memory in health and disease. *Immunol Rev.* (2018) 283:176–93. doi: 10.1111/imr.12644
98. Guy TV, Terry AM, Bolton HA, Hancock DG, Shklovskaya E, Fazekas de St. Groth B. Pro- and anti-tumour effects of B cells and antibodies in cancer: a comparison of clinical studies and preclinical models. *Cancer Immunol Immunother.* (2016) 65:885–96. doi: 10.1007/s00262-016-1848-z
99. Germain C, Gnjatich S, Dieu-Nosjean MC. Tertiary Lymphoid Structure-Associated B Cells are Key Players in Anti-Tumor Immunity. *Front Immunol.* (2015) 6:67. doi: 10.3389/fimmu.2015.00067
100. Qin Z, Richter G, Schuler T, Ibe S, Cao X, Blankenstein T. B cells inhibit induction of T cell-dependent tumor immunity. *Nat Med.* (1998) 4:627–30. doi: 10.1038/nm0598-627
101. Barbera-Guillem E, Nelson MB, Barr B, Nyhus JK, May KF, Jr., Feng L, et al. B lymphocyte pathology in human colorectal cancer. Experimental and clinical therapeutic effects of partial B cell depletion. *Cancer Immunol Immunother.* (2000) 48:541–9. doi: 10.1007/PL00006672
102. Nielsen JS, Sahota RA, Milne K, Kost SE, Nesslinger NJ, Watson PH, et al. CD20+ tumor-infiltrating lymphocytes have an atypical CD27- memory phenotype and together with CD8+ T cells promote favorable prognosis in ovarian cancer. *Clin Cancer Res.* (2012) 18:3281–92. doi: 10.1158/1078-0432.CCR-12-0234
103. Schmidt M, Bohm D, von Torne C, Steiner E, Puhl A, Pilch H, et al. The humoral immune system has a key prognostic impact in node-negative breast cancer. *Cancer Res.* (2008) 68:5405–13. doi: 10.1158/0008-5472.CAN-07-5206
104. Al-Shibli KI, Donnem T, Al-Saad S, Persson M, Bremnes RM, Busund LT. Prognostic effect of epithelial and stromal lymphocyte infiltration in non-small cell lung cancer. *Clin Cancer Res.* (2008) 14:5220–7. doi: 10.1158/1078-0432.CCR-08-0133
105. Milne K, Kobel M, Kalloger SE, Barnes RO, Gao D, Gilks CB, et al. Systematic analysis of immune infiltrates in high-grade serous ovarian cancer reveals CD20, FoxP3 and TIA-1 as positive prognostic factors. *PLoS ONE* (2009) 4:e6412. doi: 10.1371/journal.pone.0006412
106. Gu-Trantien C, Loi S, Garaud S, Equeter C, Libin M, de Wind A, et al. CD4(+) follicular helper T cell infiltration predicts breast cancer survival. *J Clin Invest.* (2013) 123:2873–92. doi: 10.1172/JCI67428
107. Ma QY, Huang DY, Zhang HJ, Chen J, Miller W, Chen XF. Function of follicular helper T cell is impaired and correlates with survival time in non-small cell lung cancer. *Int Immunopharmacol.* (2016) 41:1–7. doi: 10.1016/j.intimp.2016.10.014
108. Gu-Trantien C, Willard-Gallo K. PD-1(hi)CXCR5(-)CD4(+) TFH cells play defense in cancer and offense in arthritis. *Trends Immunol.* (2017) 38:875–8. doi: 10.1016/j.it.2017.10.003
109. Gu-Trantien C, Migliori E, Buisseret L, de Wind A, Brohee S, Garaud S, et al. CXCL13-producing TFH cells link immune suppression and adaptive memory in human breast cancer. *JCI Insight* (2017) 2:91487. doi: 10.1172/jci.insight.91487
110. Huang SC, Wei PC, Hwang-Verslues WW, Kuo WH, Jeng YM, Hu CM, et al. TGF-beta1 secreted by Tregs in lymph nodes promotes breast cancer malignancy via up-regulation of IL-17RB. *EMBO Mol Med.* (2017) 9:1660–80. doi: 10.15252/emmm.201606914
111. Veglia F, Perego M, Gabrilovich D. Myeloid-derived suppressor cells coming of age. *Nat Immunol.* (2018) 19:108–19. doi: 10.1038/s41590-017-0022-x
112. Wang C, Zhang N, Qi L, Yuan J, Wang K, Wang K, et al. Myeloid-derived suppressor cells inhibit T follicular helper cell immune response in Japanese encephalitis virus infection. *J Immunol.* (2017) 199:3094–105. doi: 10.4049/jimmunol.1700671
113. Morita R, Schmitt N, Bentebibel SE, Ranganathan R, Bourdery L, Zurawski G, et al. Human blood CXCR5(+)CD4(+) T cells are counterparts of T follicular cells and contain specific subsets that differentially support antibody secretion. *Immunity* (2011) 34:108–21. doi: 10.1016/j.immuni.2010.12.012
114. Locci M, Havenar-Daughton C, Landais E, Wu J, Kroenke MA, Arlehamn CL, et al. Human circulating PD-1+CXCR3-CXCR5+ memory Tfh cells are highly functional and correlate with broadly neutralizing HIV antibody responses. *Immunity* (2013) 39:758–69. doi: 10.1016/j.immuni.2013.08.031
115. Boswell KL, Paris R, Boritz E, Ambrozak D, Yamamoto T, Darko S, et al. Loss of circulating CD4 T cells with B cell helper function during chronic HIV infection. *PLoS Pathog.* (2014) 10:e1003853. doi: 10.1371/journal.ppat.1003853
116. Petrovas C, Koup RA. T follicular helper cells and HIV/SIV-specific antibody responses. *Curr Opin HIV AIDS* (2014) 9:235–41. doi: 10.1097/COH.000000000000053
117. Jia Y, Zeng Z, Li Y, Li Z, Jin L, Zhang Z, et al. Impaired function of CD4+ T follicular helper (Tfh) cells associated with hepatocellular carcinoma progression. *PLoS One* (2015) 10:e0117458. doi: 10.1371/journal.pone.0117458
118. Zhang H, Yue R, Zhao P, Yu X, Li J, Ma G, et al. Proinflammatory follicular helper T cells promote immunoglobulin G secretion, suppress regulatory B cell development, and correlate with worse clinical outcomes in gastric cancer. *Tumour Biol.* (2017) 39:1010428317705747. doi: 10.1177/1010428317705747
119. Zhu S, Lin J, Qiao G, Wang X, Xu Y. Tim-3 identifies exhausted follicular helper T cells in breast cancer patients. *Immunobiology* (2016) 221:986–93. doi: 10.1016/j.imbio.2016.04.005
120. Vago L, Antonacci MC, Cristina S, Parravicini C, Lazzarin A, Moroni M, et al. Morphogenesis, evolution and prognostic significance of lymphatic tissue lesions in HIV infection. *Appl Pathol.* (1989) 7:298–309.
121. Schacker TW, Nguyen PL, Beilman GJ, Wolinsky S, Larson M, Reilly C, et al. Collagen deposition in HIV-1 infected lymphatic tissues and T cell homeostasis. *J Clin Invest.* (2002) 110:1133–9. doi: 10.1172/JCI0216413
122. Nilsson J, Boasso A, Velilla PA, Zhang R, Vaccari M, Franchini G, et al. HIV-1-driven regulatory T-cell accumulation in lymphoid tissues is associated with disease progression in HIV/AIDS. *Blood* (2006) 108:3808–17. doi: 10.1182/blood-2006-05-021576
123. Estes JD, Wietgreffe S, Schacker T, Southern P, Beilman G, Reilly C, et al. Simian immunodeficiency virus-induced lymphatic tissue fibrosis is mediated by transforming growth factor beta 1-positive regulatory

- T cells and begins in early infection. *J Infect Dis.* (2007) 195:551–61. doi: 10.1086/510852
124. Pal S, Schnapp LM. HIV-infected lymphocytes regulate fibronectin synthesis by TGF beta 1 secretion. *J Immunol.* (2004) 172:3189–95. doi: 10.4049/jimmunol.172.5.3189
125. Border WA, Noble NA. Transforming growth factor beta in tissue fibrosis. *N Engl J Med.* (1994) 331:1286–92. doi: 10.1056/NEJM199411103311907
126. Zeng M, Smith AJ, Wietgreffe SW, Southern PJ, Schacker TW, Reilly CS, et al. Cumulative mechanisms of lymphoid tissue fibrosis and T cell depletion in HIV-1 and SIV infections. *J Clin Invest.* (2011) 121:998–1008. doi: 10.1172/JCI45157
127. Donovan GM, Lythe G. T cell and reticular network co-dependence in HIV infection. *J Theor Biol.* (2016) 395:211–20. doi: 10.1016/j.jtbi.2016.01.040
128. Bajenoff M, Gleichenhau N, Germain RN. Fibroblastic reticular cells guide T lymphocyte entry into and migration within the splenic T cell zone. *J Immunol.* (2008) 181:3947–54. doi: 10.4049/jimmunol.181.6.3947
129. Chai Q, Onder L, Scandella E, Gil-Cruz C, Perez-Shibayama C, Cupovic J, et al. Maturation of lymph node fibroblastic reticular cells from myofibroblastic precursors is critical for antiviral immunity. *Immunity* (2013) 38:1013–24. doi: 10.1016/j.immuni.2013.03.012
130. Siegert S, Luther SA. Positive and negative regulation of T cell responses by fibroblastic reticular cells within paracortical regions of lymph nodes. *Front Immunol.* (2012) 3:285. doi: 10.3389/fimmu.2012.00285
131. Bradley LM, Haynes L, Swain SL. IL-7: maintaining T-cell memory and achieving homeostasis. *Trends Immunol.* (2005) 26:172–6. doi: 10.1016/j.it.2005.01.004
132. Diaz A, Alos L, Leon A, Mozos A, Caballero M, Martinez A, et al. Factors associated with collagen deposition in lymphoid tissue in long-term treated HIV-infected patients. *AIDS* (2010) 24:2029–39. doi: 10.1097/QAD.0b013e32833c3268
133. Zeng M, Southern PJ, Reilly CS, Beilman GJ, Chipman JG, Schacker TW, et al. Lymphoid tissue damage in HIV-1 infection depletes naive T cells and limits T cell reconstitution after antiretroviral therapy. *PLoS Pathog.* (2012) 8:e1002437. doi: 10.1371/journal.ppat.1002437
134. Schacker TW, Nguyen PL, Martinez E, Reilly C, Gatell JM, Horban A, et al. Persistent abnormalities in lymphoid tissues of human immunodeficiency virus-infected patients successfully treated with highly active antiretroviral therapy. *J Infect Dis.* (2002) 186:1092–7. doi: 10.1086/343802
135. Zaitseva M, Blauvelt A, Lee S, Lapham CK, Klaus-Kovtun V, Mostowski H, et al. Expression and function of CCR5 and CXCR4 on human Langerhans cells and macrophages: implications for HIV primary infection. *Nat Med.* (1997) 3:1369–75. doi: 10.1038/nm1297-1369
136. Micci L, Alvarez X, Iriele RI, Ortiz AM, Ryan ES, McGary CS, et al. CD4 depletion in SIV-infected macaques results in macrophage and microglia infection with rapid turnover of infected cells. *PLoS Pathog.* (2014) 10:e1004467. doi: 10.1371/journal.ppat.1004467
137. Sattentau QJ, Stevenson M. Macrophages and HIV-1: an unhealthy constellation. *Cell Host Microbe* (2016) 19:304–10. doi: 10.1016/j.chom.2016.02.013
138. Musso T, Espinoza-Delgado I, Pulkki K, Gusella GL, Longo DL, Varesio L. IL-2 induces IL-6 production in human monocytes. *J Immunol.* (1992) 148:795–800.
139. Crotty S. Follicular helper CD4 T cells (TFH). *Annu Rev Immunol.* (2011) 29:621–63. doi: 10.1146/annurev-immunol-031210-101400
140. Chakrabarti L, Isola P, Cumont MC, Claessens-Maire MA, Hurtrel M, Montagnier L, et al. Early stages of simian immunodeficiency virus infection in lymph nodes. Evidence for high viral load and successive populations of target cells. *Am J Pathol.* (1994) 144:1226–37.
141. Durudas A, Milush JM, Chen HL, Engram JC, Silvestri G, Sodora DL. Elevated levels of innate immune modulators in lymph nodes and blood are associated with more-rapid disease progression in simian immunodeficiency virus-infected monkeys. *J Virol.* (2009) 83:12229–40. doi: 10.1128/JVI.01311-09
142. Malleret B, Maneglier B, Karlsson I, Lebon P, Nascimbeni M, Perie L, et al. Primary infection with simian immunodeficiency virus: plasmacytoid dendritic cell homing to lymph nodes, type I interferon, and immune suppression. *Blood* (2008) 112:4598–608. doi: 10.1182/blood-2008-06-162651
143. Wonderlich ER, Wijewardana V, Liu X, Barratt-Boyes SM. Virus-encoded TLR ligands reveal divergent functional responses of mononuclear phagocytes in pathogenic simian immunodeficiency virus infection. *J Immunol.* (2013) 190:2188–98. doi: 10.4049/jimmunol.1201645
144. Wijewardana V, Bouwer AL, Brown KN, Liu X, Barratt-Boyes SM. Accumulation of functionally immature myeloid dendritic cells in lymph nodes of rhesus macaques with acute pathogenic simian immunodeficiency virus infection. *Immunology* (2014) 143:146–54. doi: 10.1111/imm.12295
145. Barratt-Boyes SM, Zimmer MI, Harshyne L. Changes in dendritic cell migration and activation during SIV infection suggest a role in initial viral spread and eventual immunosuppression. *J Med Primatol.* (2002) 31:186–93. doi: 10.1034/j.1600-0684.2002.t01-1-02005.x
146. Wonderlich ER, Wu WC, Normolle DP, Barratt-Boyes SM. Macrophages and myeloid dendritic cells lose T cell-stimulating function in simian immunodeficiency virus infection associated with diminished IL-12 and IFN-alpha production. *J Immunol.* (2015) 195:3284–92. doi: 10.4049/jimmunol.1500683
147. Presicce P, Shaw JM, Miller CJ, Shacklett BL, Chougnet CA. Myeloid dendritic cells isolated from tissues of SIV-infected Rhesus macaques promote the induction of regulatory T cells. *AIDS* (2012) 26:263–73. doi: 10.1097/QAD.0b013e32834ed8df
148. Wonderlich ER, Barratt-Boyes SM. SIV infection of rhesus macaques differentially impacts mononuclear phagocyte responses to virus-derived TLR agonists. *J Med Primatol.* (2013) 42:247–53. doi: 10.1111/jmp.12064
149. Ferrando-Martinez S, Moysi E, Pegu A, Andrews S, Nganou Makamdop K, Ambrozak D, et al. Accumulation of follicular CD8+ T cells in pathogenic SIV infection. *J Clin Invest.* (2018) 128:2089–103. doi: 10.1172/JCI96207
150. Petrovas C, Ferrando-Martinez S, Gerner MY, Casazza JP, Pegu A, Deleage C, et al. Follicular CD8 T cells accumulate in HIV infection and can kill infected cells in vitro via bispecific antibodies. *Sci Transl Med.* (2017) 9:eag2285. doi: 10.1126/scitranslmed.aag2285
151. Rabin RL, Alston MA, Sircus JC, Knollmann-Ritschel B, Moratz C, Ngo D, et al. CXCR3 is induced early on the pathway of CD4+ T cell differentiation and bridges central and peripheral functions. *J Immunol.* (2003) 171:2812–24. doi: 10.4049/jimmunol.171.6.2812
152. Xu W, Joo H, Clayton S, Dullaers M, Herve MC, Blankenship D, et al. Macrophages induce differentiation of plasma cells through CXCL10/IP-10. *J Exp Med.* (2012) 209:1813–23. doi: 10.1084/jem.20112142
153. Groom JR, Luster AD. CXCR3 ligands: redundant, collaborative and antagonistic functions. *Immunol Cell Biol.* (2011) 89:207–15. doi: 10.1038/icb.2010.158
154. Khan IA, MacLean JA, Lee FS, Casciotti L, DeHaan E, Schwartzman JD, et al. IP-10 is critical for effector T cell trafficking and host survival in *Toxoplasma gondii* infection. *Immunity* (2000) 12:483–94. doi: 10.1016/S1074-7613(00)80200-9
155. Lorenzo-Redondo R, Fryer HR, Bedford T, Kim EY, Archer J, Pond SLK, et al. Persistent HIV-1 replication maintains the tissue reservoir during therapy. *Nature* (2016) 530:51–6. doi: 10.1038/nature16933
156. Moukambi F, Rodrigues V, Fortier Y, Rabezanahary H, Borde C, Krust B, et al. CD4 T Follicular Helper Cells and HIV Infection: friends or enemies? *Front Immunol.* (2017) 8:135. doi: 10.3389/fimmu.2017.00135
157. Moukambi F, Rabezanahary H, Rodrigues V, Racine G, Robitaille L, Krust B, et al. Early loss of splenic thf cells in SIV-infected rhesus macaques. *PLoS Pathog.* (2015) 11:e1005287. doi: 10.1371/journal.ppat.1005287
158. Aid M, Dupuy FP, Moysi E, Moir S, Haddad EK, Estes JD, et al. Follicular CD4 T helper cells as a major HIV reservoir compartment: a molecular perspective. *Front Immunol.* (2018) 9:895. doi: 10.3389/fimmu.2018.00895
159. Thornhill JP, Fidler S, Klenerman P, Frater J, Phetsouphanh C. The role of CD4+ T follicular helper cells in HIV infection: from the germinal center to the periphery. *Front Immunol.* (2017) 8:46. doi: 10.3389/fimmu.2017.00046
160. Lindqvist M, van Lunzen J, Soghoian DZ, Kuhl BD, Ranasinghe S, Kranias G, et al. Expansion of HIV-specific T follicular helper cells in chronic HIV infection. *J Clin Invest.* (2012) 122:3271–80. doi: 10.1172/JCI64314
161. Onabajo OO, Mattapallil JJ. Expansion or depletion of T follicular helper cells during HIV infection: consequences for B cell responses. *Curr HIV Res.* (2013) 11:595–600. doi: 10.2174/1570162X12666140225153552
162. Xu H, Wang X, Malam N, Aye PP, Alvarez X, Lackner AA, et al. Persistent simian immunodeficiency virus infection drives differentiation, aberrant

- accumulation, and latent infection of germinal center follicular t helper cells. *J Virol.* (2016) 90:1578–87. doi: 10.1128/JVI.02471-15
163. Xu H, Wang X, Malam N, Lackner AA, Veazey RS. persistent simian immunodeficiency virus infection causes ultimate depletion of follicular Th cells in AIDS. *J Immunol.* (2015) 195:4351–7. doi: 10.4049/jimmunol.1501273
164. Jacquelin B, Mayau V, Targat B, Liovat AS, Kunkel D, Petitjean G, et al. Nonpathogenic SIV infection of African green monkeys induces a strong but rapidly controlled type I IFN response. *J Clin Invest.* (2009) 119:3544–55. doi: 10.1172/JCI40093
165. Mylvaganam GH, Rios D, Abdelaal HM, Iyer S, Tharp G, Mavigner M, et al. Dynamics of SIV-specific CXCR5+ CD8 T cells during chronic SIV infection. *Proc Natl Acad Sci USA.* (2017) 114:1976–81. doi: 10.1073/pnas.1621418114
166. Kwong PD, Mascola JR, Nabel GJ. Broadly neutralizing antibodies and the search for an HIV-1 vaccine: the end of the beginning. *Nat Rev Immunol.* (2013) 13:693–701. doi: 10.1038/nri3516
167. Cubas RA, Mudd JC, Savoye AL, Perreau M, van Grevenynghe J, Metcalf T, et al. Inadequate T follicular cell help impairs B cell immunity during HIV infection. *Nat Med.* (2013) 19:494–9. doi: 10.1038/nm.3109
168. Yamamoto T, Lynch RM, Gautam R, Matus-Nicodemos R, Schmidt SD, Boswell KL, et al. Quality and quantity of TFH cells are critical for broad antibody development in SHIVAD8 infection. *Sci Transl Med.* (2015) 7:298ra120. doi: 10.1126/scitranslmed.aab3964
169. Miles B, Miller SM, Folkvord JM, Kimball A, Chamanian M, Meditz AL, et al. Follicular regulatory T cells impair follicular T helper cells in HIV and SIV infection. *Nat Commun.* (2015) 6:8608. doi: 10.1038/ncomms9608
170. Miles B, Miller SM, Folkvord JM, Levy DN, Rakasz EG, Skinner PJ, et al. Follicular regulatory CD8 T cells impair the germinal center response in SIV and *ex vivo* HIV infection. *PLoS Pathog.* (2016) 12:e1005924. doi: 10.1371/journal.ppat.1005924
171. Connick E, Mattila T, Folkvord JM, Schlichtemeier R, Meditz AL, Ray MG, et al. CTL fail to accumulate at sites of HIV-1 replication in lymphoid tissue. *J Immunol.* (2007) 178:6975–83. doi: 10.4049/jimmunol.178.11.6975
172. Xu L, Pegu A, Rao E, Doria-Rose N, Beninga J, McKee K, et al. Trispecific broadly neutralizing HIV antibodies mediate potent SHIV protection in macaques. *Science* (2017) 358:85–90. doi: 10.1126/science.aan8630
173. Havenar-Daughton C, Lindqvist M, Heit A, Wu JE, Reiss SM, Kendric K, et al. CXCL13 is a plasma biomarker of germinal center activity. *Proc Natl Acad Sci USA.* (2016) 113:2702–7. doi: 10.1073/pnas.1520112113
174. Soares EW, Nagai HM, Bredt LC, da Cunha AD, Jr., Andrade RJ, Soares GV. Morbidity after conventional dissection of axillary lymph nodes in breast cancer patients. *World J Surg Oncol.* (2014) 12:67. doi: 10.1186/1477-7819-12-67
175. van Roozendaal LM, Vane MLG, van Dalen T, van der Hage JA, Strobbe LJA, Boersma LJ, et al. Clinically node negative breast cancer patients undergoing breast conserving therapy, sentinel lymph node procedure versus follow-up: a Dutch randomized controlled multicentre trial (BOOG 2013–08). *BMC Cancer* (2017) 17:459. doi: 10.1186/s12885-017-3443-x
176. Cyr A, Gao F, Gillanders WE, Aft RL, Eberlein TJ, Margenthaler JA. Disease recurrence in sentinel node-positive breast cancer patients forgoing axillary lymph node dissection. *Ann Surg Oncol.* (2012) 19:3185–91. doi: 10.1245/s10434-012-2547-4
177. Dirix LY, Takacs I, Jerusalem G, Nikolinakos P, Arkenau HT, Forero-Torres A, et al. Avelumab, an anti-PD-L1 antibody, in patients with locally advanced or metastatic breast cancer: a phase 1b JAVELIN Solid Tumor study. *Breast Cancer Res Treat.* (2018) 167:671–86. doi: 10.1007/s10549-017-4537-5
178. Nanda R, Chow LQ, Dees EC, Berger R, Gupta S, Geva R, et al. Pembrolizumab in patients with advanced triple-negative breast cancer: phase 1b KEYNOTE-012 study. *J Clin Oncol.* (2016) 34:2460–7. doi: 10.1200/JCO.2015.64.8931

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2018 Poultsidi, Dimopoulos, He, Chavakis, Saloustros, Lee and Petrovas. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



Determination of T Follicular Helper Cell Fate by Dendritic Cells

Jayendra Kumar Krishnaswamy¹, Samuel Alsén², Ulf Yrlid², Stephanie C. Eisenbarth^{3,4} and Adam Williams^{5,6*}

¹ Bioscience, Respiratory, Inflammation and Autoimmunity, IMED Biotech Unit, AstraZeneca, Gothenburg, Sweden, ² Department of Microbiology and Immunology, Institute of Biomedicine, Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden, ³ Department of Laboratory Medicine, Yale University School of Medicine, New Haven, CT, United States, ⁴ Department of Immunobiology, Yale University School of Medicine, New Haven, CT, United States, ⁵ The Jackson Laboratory for Genomic Medicine, Farmington, CT, United States, ⁶ Department of Genetics and Genomic Sciences, University of Connecticut Health Center, Farmington, CT, United States

T follicular helper (T_{fh}) cells are a specialized subset of CD4⁺ T cells that collaborate with B cells to promote and regulate humoral responses. Unlike other CD4⁺ effector lineages, T_{fh} cells require interactions with both dendritic cells (DCs) and B cells to complete their differentiation. While numerous studies have assessed the potential of different DC subsets to support T_{fh} priming, the conclusions of these studies depend heavily on the model and method of immunization used. We propose that the location of different DC subsets within the lymph node (LN) and their access to antigen determine their potency in T_{fh} priming. Finally, we provide a three-step model that accounts for the ability of multiple DC subsets and related lineages to support the T_{fh} differentiation program.

Keywords: dendritic cell, T_{fh} cell, DC subset, DC migration, humoral response, vaccine

OPEN ACCESS

Edited by:

Shahram Salek-Ardakani,
Pfizer, United States

Reviewed by:

Vassili Soumelis,
Institut Curie, France
Dirk Baumjohann,
Ludwig-Maximilians-Universität
München, Germany

*Correspondence:

Adam Williams
adam.williams@jax.org

Specialty section:

This article was submitted to
T Cell Biology,
a section of the journal
Frontiers in Immunology

Received: 16 June 2018

Accepted: 03 September 2018

Published: 27 September 2018

Citation:

Krishnaswamy JK, Alsén S, Yrlid U,
Eisenbarth SC and Williams A (2018)
Determination of T Follicular Helper
Cell Fate by Dendritic Cells.
Front. Immunol. 9:2169.
doi: 10.3389/fimmu.2018.02169

T_{fh} CELLS IN IMMUNITY

It was initially believed that B cell activation and antibody production were regulated by the Th2 CD4⁺ T cell effector subset. Yet a principal role of effector T cells in the immune response is to deal with pathogens or tissue damage, most often outside of secondary lymphoid organs (SLOs). Indeed, upon activation, effector T cells rapidly downregulate homing receptors that keep them in the lymph node, thereby enabling migration to affected tissues. It was therefore unclear how CD4⁺ T cell help for B cells in the follicles could occur until the discovery of T follicular helper (T_{fh}) cells. T_{fh} cells are a subset of CD4⁺ T cells that function in the lymph node (LN) and spleen to promote survival, affinity maturation, and class switch recombination of B cells (1, 2). T_{fh} cells express high levels of a cell cycle inhibitor called programmed cell death-1 (PD-1), inducible T cell co-stimulator (ICOS) and the chemokine receptor CXCR5. CXCR5 expression localizes T_{fh} cells to B cell-rich areas of SLOs. These markers, in combination with the lineage-defining transcription factor BCL6, allow identification of the T_{fh} subset by flow cytometry [for review see (3)]. Discovery and characterization of the T_{fh} subset has illuminated the pathology underlying numerous diseases such as lupus as well as providing a clearer understanding of a primary cellular regulator of effective vaccine responses (4).

STAGES OF T_{fh} INDUCTION

How are T_{fh} cells induced during an immune response? It is clear that dendritic cells (DCs) and B cells must cooperate to induce and then solidify the T_{fh} fate (5–9). The DC phase occurs

over the first couple of days following T cell recognition of cognate antigen and induces a “pre-Tfh” state. In the absence of further interactions with an activated B cell, these nascent Tfh cells dissipate (10, 11). Instead, if the B cell phase ensues, a “committed” Tfh cell is produced that has the ability to enter the germinal center (GC) and in turn promote B cell proliferation, class switching, and affinity maturation. Using murine immunization models that provide high concentrations of antigen together with an adjuvant, the first DC phase can be bypassed by B cells or monocytes and plasmacytoid DCs (pDCs) (12). However, under most immunization conditions, MHCII-expressing DCs are both necessary and sufficient to induce pre-Tfh cells (6). As we will review, recent work has illuminated the nature of the DC subset capable of this step during particular types of immunizations. In both mouse and man, Tfh cells can be divided into additional subsets based on their differential expression of cytokines and chemokine receptors (13, 14). These Tfh subsets have been proposed to promote particular antibody isotypes from B cells. For example, Tfh1 cells express IFN γ , are produced during a type 1 immune response and can direct IgG2 class switching (13). DCs may also play an important role in the polarization of particular Tfh subsets. Indeed, Pattarini et al. recently showed that human Thymic Stromal Lymphopoietin (TSLP)-activated DCs seem to favor the polarization of naïve T cells into Tfh2 cells (15). However, how the different Tfh fates are induced and the particular role of DCs in guiding differentiation remains to be fully elucidated and is an area of active research.

DENDRITIC CELL SUBSETS

DCs are a heterogeneous population of cells, which can be classified as conventional DCs (cDCs) and non-conventional DCs (plasmacytoid DCs, monocyte-derived DCs, and Langerhans cells) (**Figure 1**). cDCs are the primary population responsible for naïve T cell activation and they express the transcription factor ZBTB46 (16, 17). cDCs can be further divided into two subsets based on ontogeny: type 1 cDC1s that develop in a BATF3/IRF8-dependent manner and type 2 cDC2s that are IRF4-dependent (18). These two cDC populations differ in cell surface marker expression, cytokine production, antigen processing and reside in distinct locations at steady state (18). Tissue cDC1s and cDC2s survey for infection or host damage, which if detected, induces DC migration to draining LNs. In contrast, LN-resident cDC1s and cDC2s acquire antigen that drains via lymphatics into LNs or is carried to LNs by migratory cells (19). These distinctions make each cDC subset specialized to drive particular T cell responses (20–22).

Non-conventional DCs are more diverse in their ontogeny and function. Plasmacytoid DCs (pDCs) are a unique subset that sense viral and bacterial pathogens and release high levels of type I interferons (IFN-I), stimulating both innate and adaptive immune cells. However, in comparison to cDCs, pDCs have a limited potential for antigen presentation (23). In mice there are two main monocyte subsets: inflammatory monocytes and patrolling monocytes (24). Inflammatory monocytes, including monocyte-derived DCs (moDCs), are recruited to infected tissue

where they produce inflammatory cytokines to drive local and systemic inflammation. Patrolling monocytes reside in the vasculature where they regulate homeostasis of the endothelium and promote the resolution of inflammation in damaged tissues. Finally, Langerhans cells (LCs), which have little to no expression of ZBTB46, are considered part of the macrophage lineage and are self-renewing in the epidermis. In fitting with this distinction, LCs have important functions within the tissue where they modulate the properties of other immune cell types and contribute to tissue homeostasis. However, LCs also share functional overlap with cDCs, in that they are able to acquire antigen in peripheral tissues, migrate to the lymph nodes and activate naïve T cells (25).

WHICH DC SUBSETS CONTROL Tfh DIFFERENTIATION?

While DCs have been shown to prime the first stage of Tfh cell differentiation, much debate exists around the exact DC subset that is primarily responsible for driving this response *in vivo*. A likely major reason for this controversy is differences in the approaches used. These include the site of immunization, nature of the antigen used, the dose administered, timepoint of analyses, use of antigen targeting and the kinetics of the response. Given the right experimental conditions, most DC subsets can prime Tfh cells; however, the relative contribution of each subset to the generation of humoral responses under physiologic conditions remains less clear. Below we review the current literature describing the evidence for each DC subset in Tfh priming and provide a discussion of the interpretations and caveats for each in the context of the experimental systems used.

LYMPH NODE-RESIDENT VS. MIGRATORY DCs IN Tfh PRIMING

As described previously, DCs are found both in non-lymphoid tissues like the skin, lung and gut, as well in SLOs. Both migratory and LN-resident DCs have been implicated in driving humoral responses. Most studies comparing these DC subsets were performed using antigens administered in the skin, either intradermally, or sub-cutaneously (including footpad immunization). Following immunization, antigen-bearing migratory DCs arrive in the lymph nodes after 18–24 h (26). However, injection of antigen into the footpad or ear pinnae results in an almost instantaneous delivery of antigen to the draining LN, perhaps due to the pressure induced by injection into a limited tissue space (26–31), bypassing the need for antigen delivery by migratory DCs. It is important to bear this caveat in mind when interpreting experiments using these routes of immunization. Nevertheless, this route has been used to dissect the relative contribution of LN-resident versus migratory DCs to Tfh priming in a model referred to as the “van Gogh approach” (29). In this model, antigen is delivered intradermally in the ear pinnae of mice followed by immediate resection of the injection site. This effectively eliminates migratory DC-dependent antigen transport to lymph nodes, limiting humoral

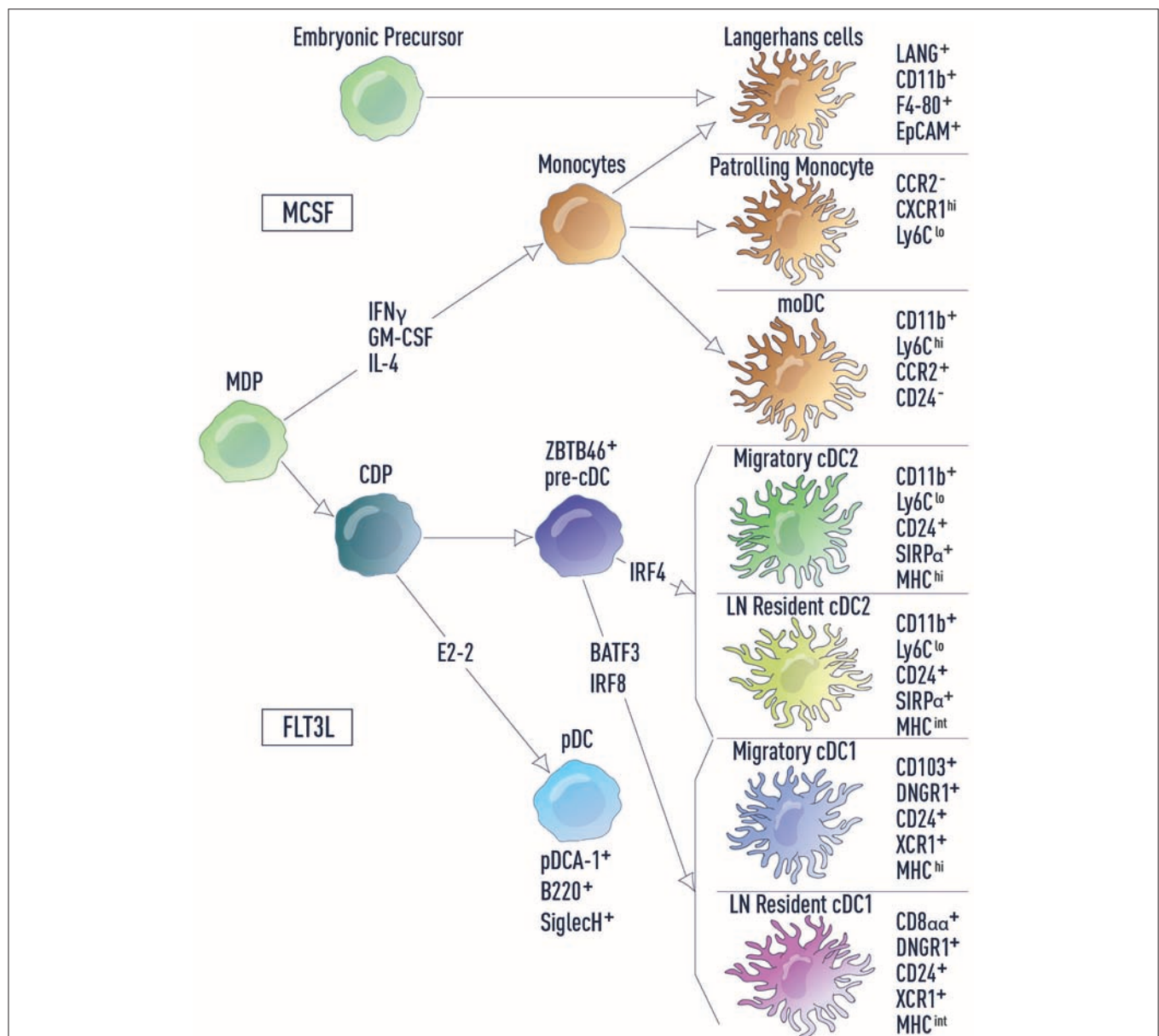


FIGURE 1 | DC subsets and related lineages. DC subsets and related lineages can be annotated based on ontogeny and function. Monocyte-macrophage DC progenitors (MDPs) differentiate into monocytes and common dendritic cell progenitors (CDPs). Monocytes further differentiate into patrolling monocytes, inflammatory monocytes (including moDCs) and Langerhans cells. Langerhans cells also develop from embryonic precursors. CDPs in turn give rise to both pDCs and pre-cDCs, which are uniquely marked by the transcription factor ZBTB46. Development of cDC2s and cDC1s from the pre-cDC is dependent on IRF4 and BATF3/IRF8, respectively. Cell surface markers used for identification of each cell type are listed.

responses to those driven by LN-resident DCs. Resecting the ear actually does not impact the total amount of antigen reaching the lymph nodes because the majority is delivered immediately via lymphatics (31). Using this approach, different studies demonstrated strikingly different results.

Intradermal vaccination in the ear pinnae with UV-inactivated influenza resulted in similar antibody responses and protection against a lethal dose of influenza in van Gogh mice as compared to controls without resection. While Tfh responses

were not evaluated, the study showed that a subset of LN-resident cDC1s and cDC2s acquire viral antigens within 40 min of immunization, migrate to the inter-follicular regions of the lymph nodes and engage with antigen-specific T cells (29). These results were reproduced in a similar study by Tozuka et al. who demonstrated that intradermally administered fluorescently-labeled antigen was acquired by a population of CD11b-expressing DCs (presumably the LN-resident cDC2s) within 30 min of immunization (28). In this study, they also

showed that ear resection immediately after (<5 s) intradermal administration of influenza HA vaccine did not impact antibody responses to the vaccine (28). Gerner et al. used advanced microscopic techniques as well as the van Gogh approach to demonstrate that LN-resident cDC2s were important for Tfh cell priming (27). Although immunization with OVA-coated beads and CpG did generate significant antibody responses in van Gogh mice, the levels of the OVA-specific IgG were significantly higher in control mice, presumably augmented by the action of migratory DCs (27). Together these studies suggest that LN-resident cDC2s are sufficient for Tfh priming under experimental conditions which circumvent the requirement for antigen delivery through DC migration.

In contrast to these three studies, also using the van Gogh model, Levin and colleagues showed that migratory DCs are required to drive Tfh and B cell responses to HIV p24-coated nano-particles and that LN-resident DCs could not support either Tfh or GC B cell dependent antibody responses (31). Using antigen encapsulated in large beads that cannot free drain into lymph, we recently demonstrated that migratory cDC2s were sufficient to induce Tfh cell priming and antibody production (30). In this model, LN-resident DCs could cooperate with migratory DCs through antigen transfer, but they were not capable on their own to induce Tfh priming.

So why these discrepancies between the studies? One explanation could be the nature of the antigen used. The studies by Woodruff et al. (29) and Tozuka et al. (28) used influenza as a model system either employing UV-inactivated influenza or influenza-derived HA protein, respectively. Resident DCs might have a higher affinity for acquiring influenza antigen and thus use of these antigens might better target LN-resident DCs. Indeed, Gonzalez and colleagues demonstrate that a SIGNR1⁺ DC subset located in the medullary sinus of the LN preferentially bound UV-inactivated PR8 strain of influenza and migrated toward the B cell follicles (32). These DCs are most likely LN resident cDC2s that are located near the lymphatic sinus (27). However, it is important to note that blocking PR8 uptake by SIGNR1⁺ DCs did not significantly impact specific antibody responses to influenza (32).

It is also possible that not all antibody responses generated against influenza are Tfh cell-dependent. In line with this, anti-influenza IgG2b/c but not IgG1 antibodies are generated in Tfh cell-deficient mice and confer protective immunity to mice upon lethal influenza challenge (33). This study suggests that while IgG1 responses require a germinal center phase, Th1 cells are sufficient to provide B cell help for extrafollicular IgG2 induction against influenza (33). Indeed, in the study by Woodruff et al. they do observe a trend for decreased anti-influenza IgG1 but not IgG2b in van Gogh mice as compared to control mice (29).

These data suggest that resident DCs can drive humoral responses under certain conditions, such as intradermal influenza vaccination (28, 29, 32). Under these conditions, a significant amount of antigen freely drains to the lymph node, bypassing the need for migratory cDCs. However, under limiting doses of antigen, LN-resident DCs may not be necessary for priming Tfh cells. A major hurdle in addressing this possibility is the lack of tools to selectively deplete LN-resident DC subsets

while keeping migratory DCs intact. Further, distinct routes of antigen transport ensure that both resident cDCs and migratory cDCs access antigen and can present antigen to naïve T cells (Figure 2). This suggests that migratory cDCs and resident cDCs might regulate distinct steps of Tfh cell differentiation, as will be discussed later.

CONVENTIONAL DCs

cDCs have been shown to play a dominant role in priming T effector responses (23). Hence, it is not surprising that they also are implicated in priming Tfh cells. The recently identified transcription factor ZBTB46 is specifically expressed by cDCs and mice encoding a diphtheria toxin receptor under the *Zbtb46* promoter (*Zbtb46-DTR*) can be used to selectively deplete cDCs *in vivo* (16, 17). Using these mice, we and others have shown the loss of T-cell dependent humoral responses in the absence of cDCs (27, 34, 35). Both cDC1s and cDC2s have been shown to drive antibody responses (see below). Identification of the most relevant cDC subset for Tfh priming is complicated by the fact that there are migratory and LN-resident subsets of cDCs and—as described above—depending on the route of immunization, the organ system studied, and type of antigen used, both migratory and resident cDCs have been implicated in priming Tfh cells.

cDC1s

Like LCs, skin-resident cDC1s, also express Langerin and hence some of the studies implicating LCs in humoral responses have also studied the role of cDC1s in mediating these responses. Using human *Langerin-DTA* mice (which lack LCs, but not cDC1s), Yao et al. demonstrated that targeting antigens to cDC1s (using antibodies against murine Langerin) in the skin is sufficient to prime Tfh cells. Further, cDC1s promoted humoral responses, albeit less efficiently than LCs in this model (36). Antigens can be efficiently targeted to cDC1s in the spleen via specific receptors such as CLEC9A or DEC-205. In two separate studies, Caminschi and colleagues demonstrate that targeting antigen (either OVA or Herpes Simplex Virus glycoprotein 1B) via CLEC9A, even in the absence of an adjuvant, primed efficient Tfh and GC B cell responses. Interestingly, they suggest that increased persistence of anti-CLEC9A mAb (and thus antigen) in the system drives enhanced CD4⁺ T cell activation and Tfh cell priming (37, 38). The authors noted that DEC-205 targeting is not as efficient in priming humoral responses especially in the absence of an adjuvant, potentially due to enhanced clearance of the mAb from circulation (38). These results were also reproduced in a separate study by Shin and colleagues (39).

In contrast, Levin and colleagues show that while depletion of LCs does partly abrogate Tfh cell and GC B cell responses to HIV p24 coated nano-particles, additional depletion of cDC1s has no further impact on these responses (31). In a similar approach, Kumamoto et al. using murine *Langerin-DTR* mice (to deplete both LCs and cDC1s) also show that cDC1s do not drive antibody responses to OVA and papain immunization in the skin (40). *Batf3*^{-/-} mice, which fail to develop cDC1s also have no defect in (and in some cases enhanced) Tfh and antibody responses to inhaled (30) and systemic antigens (34, 35). These

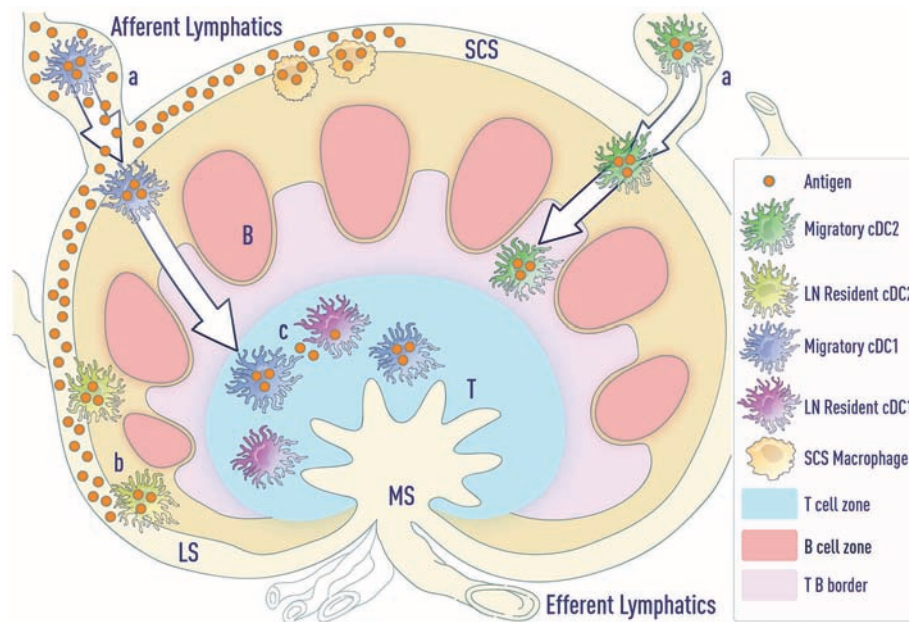


FIGURE 2 | Modes of antigen access by DCs. **(a)** Migratory cDCs (and LCs—not depicted) phagocytose antigen in the tissue and then migrate to the LNs. cDC1s migrate to the T cell zone whereas cDC2s migrate to the T-B border. **(b)** LN-resident cDC2s lining the lymphatic sinus phagocytose free draining antigen from the lymphatics. **(c)** Antigen transported by migratory DCs can be transferred to resident DC subsets in the LNs. LS (lymphatic sinus), SCS (sub-capsular sinus), MS (Marginal sinus).

results suggest that while targeting antigen to cDC1s could drive humoral responses, cDC1s are not necessary to prime Tfh and GC B cell responses to untargeted antigens, as in the case of vaccination and infection.

cDC2s

It is well documented that irrespective of the organ system, cDC2s are superior to cDC1s in their ability to prime CD4⁺ T cells rather than CD8⁺ T cells (20, 34, 41, 42). Similarly, several reports show that cDC2s are the dominant Tfh-priming DC subset (27, 30, 35, 39), potentially due to their unique localization (30, 43). Tfh cell priming occurs in the T cell-B cell border (44), which includes the interfollicular zone (IFZ). We and others have clearly demonstrated that cDC2s occupy the T-B border regions in the lymph nodes (30, 43, 45) and the spleen (35, 46–49), suggesting that this subset of DCs is ideally positioned to prime Tfh responses.

A number of studies, including ours, have investigated the role of splenic cDC2s in driving humoral responses to blood-derived antigens. Using DC-specific IRF4 knockout mice, we demonstrated that splenic cDC2s, but not cDC1s drive allo-antibody responses to transfused red blood cells (RBCs) (34). Similarly, EBI2, a G α i-coupled receptor, is required by cDC2s to position themselves in the bridging channels of the spleen, and *Ebi2*^{−/−} mice have impaired GC B cell responses to transfused sheep RBCs (48, 49). In line with this, the same authors in another report demonstrate that mice deficient in cDC2s (*Cd11c*^{Cre} *Irf4*^{−/−} and *Cd47*^{−/−}), but not cDC1s (*Batf3*^{−/−}), also have impaired Tfh responses to sheep RBCs (35). In agreement, but

using an alternative approach, Shin and colleagues showed that targeting antigens to cDC2s using antibodies against the cDC2-specific cell surface marker DCIR2 efficiently induced Tfh cell, GC B cell, and antibody responses to OVA (39, 47).

We recently reported that cDC2s also play a critical role in driving Tfh-dependent humoral responses (30). We showed that DC-specific deletion of the guanine nucleotide exchange factor *Dock8* results in impaired migration of cDC2s in the skin, lungs, and the spleen (30, 46, 50). DOCK8-deficient mice, but not *Batf3*^{−/−} (that lack all cDC1s), have defective Tfh cell responses to antigen administered sub-cutaneously, intravenously, or intranasally. As discussed previously, antigen targeting to dermal cDC1s has been shown to efficiently induce Tfh cell responses (36). However, the skin contains far fewer cDC1s than cDC2s and hence, antigen administered sub-cutaneously is not efficiently transported by cDC1s (30). In fact, most of the antigen administered sub-cutaneously is transported to the LN by migratory cDC2s. In order to compare migratory cDC1s to cDC2s, we administered antigen intranasally and found that both migratory cDC subsets efficiently transported antigens to the mediastinal LN. However, as in the skin, DC-specific DOCK8-deficient mice had impaired Tfh cell priming as well as impaired antigen-specific humoral responses to both OVA and influenza (as measured by antigen-specific IgG and weight loss in response to a lethal influenza infection). We noted similar defects in *Cd11c*^{Cre} *Irf4*^{−/−} mice which lack cDC2s. In contrast, loss of cDC1s in *BATF3*-deficient mice did not impact OVA-specific Tfh cell differentiation. In a recent study, Kumamoto et al. used *Mgl2-DTR* mice to ablate a subset of cDC2s which express CD301b.

They determined that CD301b⁺ cDC2s inhibit Tfh cell responses to antigens administered with Th2-promoting adjuvants such as papain, but not in response to Th1 adjuvants like CpG (40). Although the mechanism remains to be determined, this work highlights that the major DC subsets outlined in **Figure 1** are likely more heterogeneous than currently appreciated.

While these reports suggest that there is some consensus about the proficiency of cDC2s in Tfh cell priming, discrepancies do exist, especially regarding the role for migratory versus LN-resident cDC2s. In an elegant study using an advanced multi-parameter microscopic technique called histo-cytometry, Gerner and colleagues showed that migratory cDC2s occupy the IFZ (part of the T-B border) in inguinal LNs, while resident cDC2s are found in the lymphatic/medullary sinus regions (27). Based on this observation, one could hypothesize that migratory cDC2s are better positioned to drive Tfh responses; however, this study found that migratory cDC2s were not required for Tfh induction. Again, this was using methods of immunization that might bypass normal trafficking routes such as intra-auricular injection (31). To distinguish the migratory cDC2s from resident cDC2s (and cDC1s), we used OVA-encapsulated beads that cannot free drain to the lymph nodes via the lymphatics and hence can only be transported by migratory cDCs. Administration of these beads to DOCK8-deficient mice resulted in impaired Tfh cell responses, re-emphasizing the inability of cDC1s to drive these responses. In BATF3-deficient mice, cDC2s are unaffected and administration of beads to these mice restricts antigen to migratory cDC2s. Tfh cell frequencies in BATF3-deficient mice were normal as compared to WT controls, indicating that migratory cDC2s are sufficient to prime Tfh cell responses (30).

The overall conclusion from these studies is that cDC2s play a dominant role in priming Tfh cells. However, as will be discussed next, other non-conventional DCs likely partner with cDC2s to promote early phases of T cell activation. The discrepancies stated above, however, suggest a greater level of heterogeneity than currently appreciated among cDC2s. Future studies to selectively target different subsets of cDC2s including migratory, resident and CD301b-expressing cells will help clarify positive and negative influences on Tfh differentiation.

NON-CONVENTIONAL DCs

Monocyte-Derived DCs

Using the van Gogh model Levin et al. show that while ear resection does not impair the frequency of monocyte populations in the draining lymph node, it does result in impaired Tfh responses to HIV p24 coated nano-particles (31). Similarly, Kumamoto et al. showed that depletion of monocyte-derived DCs using anti-Gr1 antibodies also had no impact on Tfh responses (40). Together, these results suggest that monocyte-derived DCs are not required for priming Tfh cells.

However, using different models, other groups have come to the opposite conclusion. Barbet et al. show that Tfh responses to intraperitoneal *E. coli* vaccination are TRIF-dependent, and driven by CD11c⁺ CX3CR1⁺ “patrolling” monocytes. They demonstrate that TRIF-dependent Tfh priming is unaffected in the absence of cDCs (using *Zbtb46-DTR* mice)

or inflammatory monocytes (*Ccr2*^{-/-} mice) (51). Immunization with a combination of CpG-B and incomplete Freund's adjuvant (IFA) results in higher frequencies of Tfh cells as compared to IFA alone (52). Using *Ccr2*^{-/-} or *Cx3Cr1*^{-/-} mice, Chakarov et al. generated mice with deficiencies in moDCs but not cDCs to demonstrate that the enhanced Tfh response induced by CpG-B is driven by IL-6-producing moDCs (52). However, given that a significant frequency of Tfh cells were induced in the absence of moDCs (52), one conclusion could be that, while cDCs play a dominant role in priming Tfh responses, moDCs enhance this response via IL-6 production. Interestingly, Germain and colleagues demonstrated that administration of high doses of DT to *Zbtb46-DTR* mice triggers infiltration of CD11b-expressing monocytic populations that are distributed throughout the lymph node (27). Thus, while these cells may not form part of the canonical Tfh differentiation pathway, these results together indicate that under certain conditions, moDCs can occupy similar functional niches as cDC1s and cDC2s and can help prime Tfh responses.

Langerhans Cells

The absolute requirement of LCs for humoral responses has been addressed by several studies using Langerin-diphtheria toxin receptor mice (31, 40, 53). To delineate which migratory DC subsets play a role in Tfh responses, Levin et al. used *Langerin-DTR* mice to deplete Langerin-expressing DCs i.e., LCs and cDC1s in the skin (31). Post DT treatment, LCs remain depleted for more than 2 weeks while cDC1s are replenished within 1 week. They employed this differential response to generate mice lacking only LCs (DT administered 2 weeks prior to immunization) or both LCs and cDC1s (DT administered 2 days prior to immunization). Loss of either LCs alone or both LCs and cDC1s decreased but did not abrogate Tfh and antibody responses to HIV p24-coated nano-particles that were administered in the absence of any other adjuvant. The authors conclude that while LCs do play a role in Tfh-dependent B cell responses, migratory cDC2s also contribute to humoral responses in the skin (31). Corroborating these results, in a model using OVA and the adjuvant papain, Kumamoto et al. demonstrate that LCs are not required for humoral responses to cutaneous antigens (40).

In an earlier study using the same *Langerin-DTR* mice, Zimara et al. demonstrated that loss of LCs results in impaired Tfh induction and early antibody responses (day 10) to *Leishmania major* infection (53). However, overall *Leishmania*-specific humoral responses (i.e., day 40 post-infection) remain unaffected. The study further showed that the size of GC is decreased in mice lacking LCs. These impaired responses are, however, restricted to *Leishmania* infection and not seen with other T-dependent antigens like DNP-KLH (with the adjuvant aluminum hydroxide). The authors suggest that *Leishmania* infection, unlike DNP-KLH and alum, does not lead to maturation of cDCs and under these circumstances, LCs drive humoral responses (53).

Yao et al. used transgenic mice expressing human Langerin (*huLangerin*), specifically in LCs but not in other DCs (i.e., dermal cDC1s, where murine Langerin is expressed) (36).

Using monoclonal antibodies specific to human Langerin, the authors demonstrate that targeting antigens to LCs *in vivo* efficiently induces antigen-specific Tfh cell responses. The response generated was dose-dependent and was only generated against foreign antigens (and not self-antigens like MOG peptide). Targeting LCs either in the skin and LNs (systemic administration) or in the skin alone (topical application) efficiently induced B cell responses including GC B cell formation and a protective humoral response against lethal influenza challenge (36). It is interesting to note that as compared to previously published reports (7, 44), the kinetics of the response generated by targeting LCs is slightly delayed, i.e., the peak of the Tfh cell response observed is around day 7 and the peak of GC B cell expansion is around day 14 (36). One potential explanation for this is that LCs are known to have significantly slower migration kinetics with their numbers peaking 3–4 days post-immunization (even under inflammatory conditions) as opposed to cDCs that reach the draining cutaneous lymph nodes 18–24 h post-immunization (50, 54, 55).

In the work by Yao et al. LCs were shown to primarily drive humoral responses when antibodies to Langerin were administered without an adjuvant (36). Under these circumstances, the lack of adjuvant would fail to efficiently induce maturation and migration of migratory cDCs— a prerequisite for their ability to induce effective T cell responses. Indeed, even under inflammatory conditions, expression of co-stimulatory molecules remain unchanged on LCs that migrate to the LN, suggesting that under steady state, LCs have a mature phenotype (54). In contrast, migratory cDCs have higher expression of co-stimulatory molecules and emigrate in greater frequencies upon maturation (30, 56) possibly indicating that these cells play a dominant role under inflammatory conditions such as in the case of an infection or vaccine response. These results together suggest that LCs can drive humoral responses to low-abundance, weakly immunogenic antigens that do not efficiently induce cDC maturation (36, 53).

DC-DEPENDENT FACTORS REGULATING EARLY Tfh DIFFERENTIATION

Early differentiation signals required for Tfh cells have been extensively characterized. Signals that function early in the Tfh differentiation process, and that are independent of B cells, have frequently been ascribed to DCs. However, it is important to note that there is still limited evidence that directly proves that these are DC-unique factors. This is further complicated by the fact that multiple DC subsets could play a role in the differentiation process.

DC Maturation and Pattern Recognition Receptors

DCs must undergo a maturation process for the induction of a productive adaptive immune response. Almost 30 years following Charles Janeway's proposal, several PAMPs (pathogen-associated molecular patterns) or DAMPs (danger-associated molecular patterns) have been identified that are detected by the immune

system via specific groups of germ-line encoded receptors called Pattern Recognition Receptors (PRRs). These include Toll-like receptors (TLRs), C-type lectin receptors (CLRs), NOD-like receptors (NLRs), RIG-I-like receptors (RLRs) and AIM2-like receptors (ALRs). Engaging these receptors results in DC maturation and thereby an effector T cell response (57).

TLR agonists have widely been used in induction of Tfh responses. Notably, TLR3 (39, 58), TLR4 (5, 30, 39, 58) and TLR9 (27, 40, 52) agonists have been shown to effectively drive Tfh responses in both mice and humans. Kumamoto and colleagues show that the immunosuppressive effects of CD301b⁺ cDC2s is only seen using “Th2-type” adjuvants like papain and that engagement of TLRs like TLR4 and TLR9 overcomes the Tfh-inhibiting capacity of this DC subset (40). In a recent study, Ugolini et al. demonstrate that TLR8 on human monocytes senses microbial viability by binding to bacterial mRNA. The activation of monocytes by this special class of PAMPs called “vita-PAMPs,” results in production of IL-12 which in turn drives BCL6 expression and IL-21 production by CD4 T cells (59). Barbet et al. reported that in mice, bacterial viability is detected by CX3CR1⁺ monocytes via a TRIF-dependent mechanism. The downstream Type I IFN response along with inflammasome activation drives Tfh differentiation (51). While the role of IFN signaling in driving Tfh responses is discussed later, it is important to note that TLR3 and TLR4 agonists also drive Tfh responses by inducing an autocrine Type I IFN signal in DCs (58). Using human monocyte-derived DCs, Schmitt et al. compared Tfh inducing capacities of different TLR agonists and show that TLR4, TLR5, and TLR7/8, but not TLR2, activation induces IL-21 production from CD4⁺ T cells, with TLR4 being the most potent followed by TLR5 and TLR7/8 (5).

Monoclonal antibodies against C-type lectin receptors (CLRs) have been used to target antigens to specific DC subsets *in vivo* and, as described previously, several studies have employed this approach to study the Tfh-priming capacities of different DC subsets. Targeting antigen to certain C-type lectin receptors is alone sufficient to prime Tfh cells and does not require additional adjuvants. For example, targeting LCs via the CLR Langerin does not result in LC activation but is sufficient to drive Tfh cell differentiation *in vivo* (36). Similarly, Caminschi and colleagues demonstrate that targeting antigen to cDC1s via the CLR CLEC9A induces a robust Tfh cell response (37, 38) and this response is not augmented by co-administering the TLR9 agonist, CpG (38). In contrast, Shin et al. report that effective Tfh cell differentiation is observed when antigen is targeted to cDC2s via the CLR DCIR2 in the presence of TLR3 (Poly I:C) or TLR4 (LPS) agonists (39).

There are a limited number of studies addressing the role of other groups of PRRs in driving Tfh differentiation. While the role of NLRs in priming Tfh cells has not been directly addressed, alum, a potent activator of the NLRP3 inflammasome (60, 61), has been used as an adjuvant in several studies (6, 62, 63). Further, IL-1 β , an effector cytokine produced downstream of inflammasome activation (57), also plays a role in priming Tfh cells, as will be discussed later. Regarding the role of RLRs, one study demonstrated that co-administering influenza antigens with 5' ppp-double-stranded RNA, a RIG-I ligand, enhances Tfh

differentiation and antibody responses to influenza via a Type I IFN-dependent mechanism (64).

Antigen Presentation

The strength and duration of antigen presentation plays a critical role in determining the outcome of CD4⁺ T cell responses, i.e., T effector cells versus Tfh differentiation (65). Using a pigeon cytochrome C (PCC) model, Fazilleau et al. demonstrated that T cells with higher antigen affinity preferentially differentiate into Tfh cells (66). Tfh cells have stronger affinity for peptide-MHC II complexes and a more restricted TCR repertoire as compared to T effector cells (66). In addition to TCR affinity, increased TCR signals (using high antigen concentrations) are required for maximal IL-21 production (66). Moreover, increasing the antigen dose (11) or gradually increasing antigen administration over 2 weeks (67) boosts the generation of both Tfh and GC B cells. Thus, antigen dose is tightly linked to both Tfh induction and the magnitude of GC responses. In contrast, both high and low affinity antigen-specific T cell clones equally differentiate into Tfh cells in mice immunized with Friend's virus (68). This study used a chronic retroviral infection model and the authors suggest that the differences in their study as compared to Fazilleau et al. is probably due to antigen availability. Thus, they hypothesize that under conditions of limiting antigen availability, high TCR avidity would drive Tfh cell differentiation (68). However, TCR avidity, as in the case of the previous study, does impact IL-21 production, suggesting that some features of Tfh differentiation are indeed TCR-intrinsic (68).

Nevertheless, the contrasting results of these studies suggest that simple affinity of TCR to peptide-MHC II complexes, i.e., the receptor occupancy model, would not explain Tfh versus T effector cell differentiation. An alternative kinetic proofreading model suggests that the duration of interaction between TCR and peptide MHC II complexes (i.e., DC-T cell interaction times) is a better predictor of the outcome of the T cell response. Using single cell clones, Tubo and colleagues elegantly provide support for this model. They show that longer dwell time between TCR and peptide-MHCII, rather than TCR affinity, preferentially drives Tfh cell differentiation (69). In a recent study, Benson et al. visualized this process *in vivo* to show that the time of antigen presentation by DC to CD4⁺ T cells is critical for Tfh differentiation *in vivo* (7). Immunizing mice sub-cutaneously with 200 nm sized antigen-coated nanoparticles efficiently primed Tfh responses and protective antibody responses to influenza. Using multiphoton imaging, DC and antigen-specific CD4⁺ T cell interactions were imaged *in vivo*. The authors defined 3 stages of DC-T cell interaction over the course of the immune responses: Stage 1 (0–8 h), Stage 2 (12–24 h), and Stage 3 (48–72 h) post-immunization. Interactions longer than 10 min between DC-CD4⁺ T cells at Stage 3 were required for efficient Tfh cell priming. Disrupting MHCII-TCR binding at this stage impaired Tfh cell frequencies, suggesting that sustained antigen presentation is required for Tfh cell differentiation (7). These results together indicate that DC subsets that stably express antigen-MHCII complexes are probably superior at priming Tfh cells. Since cDC2s are more efficient than cDC1s in processing antigen for MHCII, this could

explain why this subset seems to be more effective in Tfh priming, as discussed above (20, 70). However, experimental models in which antigen is specifically targeted to cDC1s could compensate for this difference and thereby enhance the ability of cDC1s to promote Tfh differentiation (37, 38).

Co-stimulatory Molecules

Tfh priming requires a variety of co-stimulatory molecules including B7 family members, CD40L, OX40L, and ICOSL (63). The most extensively studied co-stimulatory molecule with regards to Tfh priming is OX40L. However, studies have revealed, at least in part, that CD28 engagement and/or CD40L engagement leads to upregulation of OX40 on T cells (63).

Early reports demonstrated that CD28-deficient mice have impaired GC and humoral responses (71). Mice overexpressing CTLA4 (mCTLA4-Hy1 transgene), the inhibitory ligand for CD28, also showed similar impairment in T-dependent B cell responses (72). However, Lane and colleagues in later studies demonstrated that the loss of these humoral responses was primarily due to impaired OX40 expression. OX40 is upregulated on naïve T cells following CD28 activation and activation of T cells by OX40L promotes expression of IL-4 and CXCR5 (73, 74). Interestingly, constitutive expression of OX40L by DCs (using CD11c-OX40L transgenic mice) leads to increased Tfh cell differentiation; however, this is also dependent on CD28 signaling (74). A recent study by Watanabe et al. using Cre-mediated deletion of CD80 and CD86 in DCs illustrated that CD28 signaling at the stage of T-DC interaction is critical for initial priming and expansion of T cells (75). In contrast, loss of CD80 and CD86 in the B cell compartment did not affect the generation of Tfh cell or GC B cells nor humoral responses in terms of affinity maturation and serum IgG levels (75). Collectively, these studies demonstrate a critical role of DCs in delivering CD80/CD86 co-stimulatory molecules during pre-Tfh differentiation for optimal Tfh and GC responses.

Patients with CD40L deficiency as well as CD40-deficient mice show impaired Tfh cell frequencies (76, 77). Given that CD40 activation leads to OX40L expression on both murine and human DCs (78, 79), the Tfh defects seen in the absence of CD40 could indeed be a downstream effect of abrogated OX40/OX40L signals. In line with this, Fillatreau et al. report that *Cd40*^{−/−} mice, like *Ox40*^{−/−} mice, have impaired accumulation of T cells in follicles. CD40 is required for CXCR5 expression in T cells (80). Restoring CD40 expression in DCs but not B cells, either using mixed bone marrow chimeras or by adoptive transfer of CD40⁺ DCs, restores this response in *Cd40*^{−/−} mice. Furthermore, treating *Cd40*^{−/−} mice with OX40L-huIgG1 fusion proteins readily rescues CD4⁺ T cell migration into follicles (80). However, administration of agonistic OX40 antibodies during LCMV infection diverts Tfh differentiation to T effector differentiation by inducing Blimp-1 expression (81), suggesting that the role of OX40 signaling in Tfh differentiation is context-dependent.

These results suggest that Tfh priming is primarily regulated by OX40 signaling downstream of CD28 and CD40L. However, Akiba et al. compared the effects of all three co-stimulatory molecules on Tfh priming *in vivo* and clearly demonstrate

that while CD28 and CD40 are indeed required for Tfh cell differentiation and GC B cell responses, the requirement for OX40 signaling is strain- and immunization site-specific (76). The authors immunized various mouse strains, including BALB/c and C57BL/6, at different sites and compared OX40 expression on Tfh cells in the spleens and in skin-draining LNs. They show that OX40 blocking antibodies only impair GC and Tfh responses in LNs of C57BL/6 mice but not in BALB/c mice, possibly since OX40L expression is observed only in LN but not splenic Tfh cells in C57BL/6 mice post-immunization (76). The requirement for CD28, beyond OX40 upregulation, in early Tfh cell responses is further demonstrated in a study by Smith and colleagues (82). *Cd28^{flox/flox} Ox40^{Cre/+}* mice were used to block CD28 signaling after T cell priming and expansion. The authors show that these mice have reduced frequencies of Tfh and GC B cells in response to intranasally administered influenza A, as compared to control mice. Loss of CD28 in activated T cells, results in increased apoptosis and impaired BCL6 and ICOS expression in Tfh cells (82). These results suggest that persistent CD28 stimulation, beyond early naïve T cell activation, is required for Tfh cell differentiation and maintenance as well as functional humoral responses (82).

A recent study by Tahiliani et al. demonstrates that in a murine model of vaccinia virus infection, OX40-deficient mice have impaired Tfh and B cell responses (83). Blocking OX40L during and after Tfh cell generation leads to a significant reduction in Tfh, GC Tfh and GC B cell frequencies (83). Finally, OX40L-expressing DCs were seen in bridging channels in the spleen and also co-localized with OX40⁺ T cells, suggesting that cDC2s could in part provide some of these signals during Tfh cell priming (83). Further, TSLP-activated human DCs prime IL-21- and CXCL13-producing CXCR5⁺ PD-1⁺ Tfh cells *in vitro*. Blocking OX40L, but not ICOSL, in this system reduces IL-21 and CXCL13 production. It is important to note that while BCL6 expression is reduced in CXCR5^{hi} PD1^{hi} cells, blocking OX40L in this system does not reduce Tfh cell frequencies (15). Together, these results indicate that the requirement for OX40L during Tfh cell differentiation is not absolute, but under specific conditions, could regulate certain facets of Tfh cell differentiation.

ICOS signaling plays a central role in Tfh cell priming. ICOS-deficient mice and humans have impaired Tfh cell and GC B cell frequencies, reduced T cell localization to follicles and impaired humoral responses (76, 77). Roquin 1 and Roquin 2 are RNA-binding proteins that play an important role in post-transcriptional repression of ICOS expression. Combined loss of Roquin 1 and Roquin 2 (84, 85), or loss of mir-146a (also a negative regulator of ICOS) (86) specifically in T cells, increases ICOS expression, resulting in spontaneous accumulation of Tfh and GC B cells in mice (84–86). B cell-specific ablation of ICOSL results in a similar loss of Tfh cell differentiation as ICOSL knockout mice, which suggests that ICOS signaling primarily plays a role during the B cell phase of Tfh priming (87). Similarly, blocking ICOSL *in vitro* did not impair human Tfh cell differentiation by TSLP-activated DCs (15). In contrast, Choi et al. demonstrate that early ICOS signaling is required for BCL6 expression and Tfh cell commitment as *Icos*^{-/-} T cells failed to differentiate into Tfh cells as early as day 3

post-immunization (88). The authors also show that loss of B cells had no impact on Tfh cell frequencies 3 days post-immunization, suggesting that this stage was B cell-independent. Further, adoptive transfer of antigen-loaded DCs was sufficient to induce BCL6 and CXCR5 expression in T cells confirming that this early stage of Tfh cell differentiation is indeed DC-dependent. The impaired differentiation of *Icos*^{-/-} T cells to Tfh cells early in the response suggests that ICOSL signaling by DCs is critical for Tfh cell priming. Further, ICOS signaling during this first stage is required for BCL6 expression by Tfh cells, which the authors show in turn is critical for CXCR5 expression (88). Together, these findings highlight that ICOS is important for multiple stages of Tfh differentiation and is provided by both DCs and B cells.

Finally, NOTCH signaling also regulates Tfh cell differentiation. Loss of NOTCH 1 and 2 specifically in CD4⁺ T cells leads to reduced Tfh cell frequencies, impaired IL-4 production by Tfh cells and concomitantly, reduced GC B cell and IgE responses (89, 90). While NOTCH signaling controls IL-4 production by Tfh cells (90), NOTCH-deficient Tfh cells also fail to downregulate BLIMP1 (PRDM1) or upregulate BCL6, Cmaf, and IL-21, in an IL-4-independent manner (89). DC-specific deletion of the NOTCH ligand, the E3 ubiquitin ligase Mind bomb1 (MIB1) also impairs early Tfh cell differentiation. Tfh cell frequencies in these mice are eventually comparable to controls at later stages of the response indicating that requirement for DC-derived NOTCH signals is not absolute. The authors also demonstrate that depletion of NOTCH ligands on B cells and follicular DCs has no impact on Tfh cell priming (90).

Cytokines

The cytokine milieu in SLOs plays a critical role in polarizing T cells either toward Tfh or other T effector fates. Depending on the PAMPs or DAMPs associated with the antigen encountered, DCs secrete cytokines that could significantly influence the outcome of the T cell response. Given that different DC subsets preferentially express distinct cytokines, this may specialize them for driving the polarization of different T cell subsets. Further, as discussed previously, distinct DC subsets occupy unique niches within the SLOs; therefore, a combination of DC-intrinsic differences in cytokine production in combination with cells in the niche could favor Tfh differentiation versus other T effector fates.

IL-6

In the mouse, IL-6 is one of the first cytokine signals to influence the early stages of Tfh differentiation. IL-6 signals via STAT3 and this pathway induces early expression of BCL6 in T cells (91, 92). Deficiency of IL-6 or STAT3 impairs Tfh differentiation and antibody responses *in vivo* (91, 93). DCs produce copious amounts of IL-6 in response to stimulation with various PAMPs or CD40 activation (2). Adoptive transfer of antigen-pulsed IL-6-sufficient DCs but not IL-6-deficient DCs leads to efficient antibody responses *in vivo* (93). DC-specific deletion of *Blimp1* in mice results in a spontaneous lupus-like phenotype characterized by increased Tfh cell frequencies and

autoantibody production (94). The authors demonstrate that BLIMP1 deficiency causes increased IL-6 production by DCs. Further, *Il-6* heterozygous DC-specific *Blimp1*-deficient mice (*Il-6*^{+/-} *DCBlimp1*^{ko}), in which IL-6 expression is no longer elevated, have reduced GC and Tfh cell responses compared to *DCBlimp1*^{ko}, suggesting that DC-derived IL-6 drives the responses observed in these mice (94). In a subsequent study, Kim et al. defined IL-6-dependent and -independent pathways by which BLIMP1 also regulates the expression of Cathepsin S, an endolysosomal protease which influences antigen processing, suggesting this as an additional mechanism for the observed lupus-like phenotype (95). They showed that DCs from BLIMP1-deficient mice induced IL-21 expression in co-cultured T cells and that this is abrogated in the presence of a Cathepsin S inhibitor (95).

These results indicate that DCs could indeed be the source of IL-6 for Tfh cell differentiation. However, other studies suggest that, rather than driving early Tfh cell differentiation, DC-derived IL-6 instead fine-tunes the phenotype of newly primed Tfh cells, with other cell types producing the IL-6 that is required for early Tfh differentiation. Using adoptively transferred IL-6-deficient antigen-pulsed DCs, Andris and colleagues show that DC-derived IL-6 has no impact on Tfh cell frequencies or CXCR5, PD1 and BCL6 expression (96). This is in line with a report from Chen et al. who, using mixed bone marrow chimeras, report that IL-6 deficiency in radio-resistant (such as stromal cells) but not radio-sensitive hematopoietic cells (such as DCs), impacts CXCR5 and BCL6 expression in Tfh cells (62). However, Andris and colleagues also show that IL-6-mediated STAT3 signaling suppresses GATA3 expression in Tfh cells. Loss of DC-derived IL-6 results in decreased IL-21 and increased IL-4 production by Tfh cells, resulting in increased IgE responses (96). These results indicate that IL-6 production from stromal cells as well as DCs have non-redundant functions during Tfh cell differentiation.

IL-12

IL-12 has been shown to play a critical role in Tfh cell differentiation in human T cells (5). Triggering of certain pattern recognition receptors like TLRs induces IL-12 production by DCs (2). In two separate studies, IL-12, and to a lesser extent IL-23, were able to induce IL-21 from human CD4⁺ T cells activated *in vitro* (5, 97). IL-21-expressing T cells express CXCR5 and ICOS and promoted B cell help *in vitro* (5, 97). Similarly, allogenic stimulation of T cells with DCs exposed to different heat-killed bacteria, resulted in IL-21 expression by T cells which in turn regulated antibody production by B cells *in vitro*. This process is IL-12-dependent, as inhibiting IL-12 *in vitro* abrogated these responses (5). Human cDC2s produce higher levels of IL-12 and IL-6 as compared to cDC1s in response to a variety of TLR ligands (98, 99). This might explain, in part, why TSLP-activated human cDC2s are superior to cDC1s in activating and inducing IL-21 production from T cells (15). Using adoptive transfer of DCs, Andris and colleagues demonstrated that loss of IL-12 does not impact murine Tfh cell differentiation (96). In contrast to human cDC2s, murine cDC2s actually produce less IL-12 (30, 100). Therefore, the role of IL-12 appears to be different

in human vs. murine Tfh-DC interactions and further work remains to be done on what accounts for these species-specific effects.

Type I IFN

Type I IFN and IL-27 modulate Tfh cell differentiation. Interferon-alpha/beta receptor-deficient (*Ifnar*^{-/-}) mice have impaired Tfh cell and antibody responses as compared to control mice when immunized with NP-OVA and LPS (58). IFNAR deficiency in either DCs or radio-resistant cells also results in reduced frequencies of Tfh cells. Further, *Ifnar*-deficiency in DCs results in reduced IL-6 production (58). As discussed previously, IL-6 production from stromal cells and DCs seems to non-redundantly impact Tfh cell differentiation and these data further support this hypothesis.

While IFN-induced IL-6 production provides one potential mechanism, other pathways downstream of IFNAR signaling have also been shown to drive Tfh cell responses. Gringhuis et al. demonstrate that autocrine IFNAR signals in human DCs can promote IL-27 production that in turn drives Tfh cell differentiation (101). They show that treating human DCs with fucose, an agonist of the C-type lectin receptor DC-SIGN, activates IKKε, a member of the non-canonical IKK kinase family. This pathway synergizes with an autocrine Type I IFN signal to drive IL-27 production. The IL-27 produced by the DCs enhances BCL6 and IL-21 expression in T cells (101). In line with this, using *Il27ra*^{-/-} mice, Batten et al. demonstrated that IL-27 promoted IL-21 production and Tfh cell survival (102). A recent study by Blander and colleagues also demonstrated an alternative pathway by which Type I IFN regulated Tfh differentiation (51). They demonstrated that autocrine IFNAR signals promoted Caspase 1- and Caspase 11-mediated production of IL-1β by DCs. In T cells, IL-1β signals via IL1R1 and drives expression of BCL6, CXCR5, and ICOS. In addition, IFNAR signaling in Tfh cells directly induces production of IL-21 (51). However, this study also shows that CX3CR1⁺ CCR2⁻ monocyte-derived DCs and not cDCs drive these Tfh responses (51). The presence of multiple pathways by which IFNAR signaling regulates Tfh cell differentiation raises the possibility that depending on the antigen encountered, different DC subsets could utilize alternative IFNAR-dependent pathways to prime T-dependent B cell responses.

IL-2

IL-2 is a negative regulator of Tfh cell differentiation. IL-2 induces BLIMP-1 expression in T cells which suppresses BCL6 and downregulates CXCR5 (1). Further, IL-2-mediated mTORC1 activation of AKT also suppresses Tfh cell differentiation (103). Under certain stimuli, DCs have been shown to produce IL-2, a process that is counter-productive for Tfh cell priming (2). However, a recent study by Cyster and colleagues demonstrates that certain DC subsets like cDC2s express the IL-2 receptor alpha chain, CD25 (35). However, cDC2s do not respond to IL-2, as seen by STAT5 phosphorylation. The authors suggest that both soluble and secreted CD25 expression by cDC2s creates a “cytokine-sink” for IL-2. Limiting concentrations of IL-2 in

the T-B border makes this niche then favorable for Tfh cell differentiation (35). However, in our studies, while we did find higher expression of CD25 on lung cDC2s, we could not find evidence supporting the role of DC-dependent CD25 expression on Tfh responses (30). Given that other cells in the T-B border including activated B cells express CD25 (104), we hypothesize that multiple cell types could cooperate to create an effective IL-2 sink.

THE THREE-STEP DIFFERENTIATION MODEL

Spatio-temporal distribution of lymphocyte subsets within the SLOs are critical for efficient Tfh cell priming. Based on the literature reviewed here, the following conclusions can be drawn regarding DC subsets and Tfh priming.

1. DC subsets occupy distinct regions within lymphoid organs. Migratory cDC1s, resident cDC1s and LCs are found within the T cell zones, migratory cDC2s are located in the T-B border region (including the IFZ) and resident cDC2s reside in the lymphatic sinuses (30, 45).
2. Excluding antigens from certain DC subsets or depletion of specific DC subsets does not impair naïve T cell activation (27, 30, 31), suggesting that a redundancy exists in this step of T cell priming.
3. Antigen availability in SLOs determines which antigen presenting cells (APCs) are able to support Tfh priming. Decreasing antigen concentration progressively increases the dependency on APCs that are more effective in priming Tfh cells (**Figure 3**).
4. Distinct DC subsets can sequentially prime T cell responses (26).
5. Early after activation, naïve CD4⁺ T cells differentiate into BCL6⁺ pre-Tfh cells (9) and migrate to the T-B border - a process regulated by multiple factors including CCR7 (105), CXCR5 (44, 62, 105), and EBI2 (35). Pre-Tfh cells differentiate in the T-B region to Tfh cells (44). This process is initially B cell-independent and DC-dependent (6, 44, 63, 88).
6. Tfh cells interact with antigen-bearing activated B cells and mature into effector Tfh cells (1).

Based on these data, we propose a three-step model for Tfh cell differentiation under conditions that deliver sufficient free draining antigen to LNs (e.g., high antigen doses, footpad or intra-auricular immunization, or infection within the LN) (**Figure 4**).

1. **Antigen transport and naïve T cell activation:** DCs can access antigens in three ways (**Figure 2**):
 - Migratory cDCs (and LCs) phagocytose antigen *in situ* and then migrate to the LNs
 - LN-resident cDC2s lining the lymphatic sinus endothelium phagocytose free draining antigen from the lymphatics
 - Antigen transported by migratory DCs can be transferred to resident DC subsets in the LNs

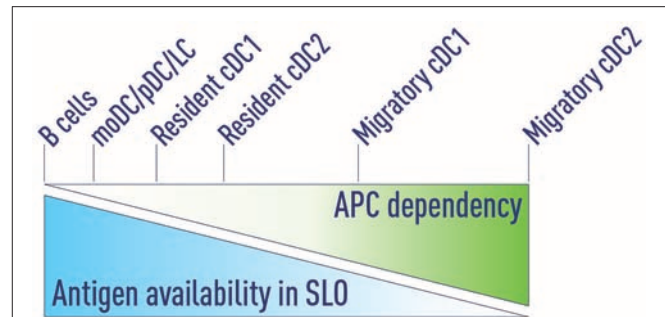


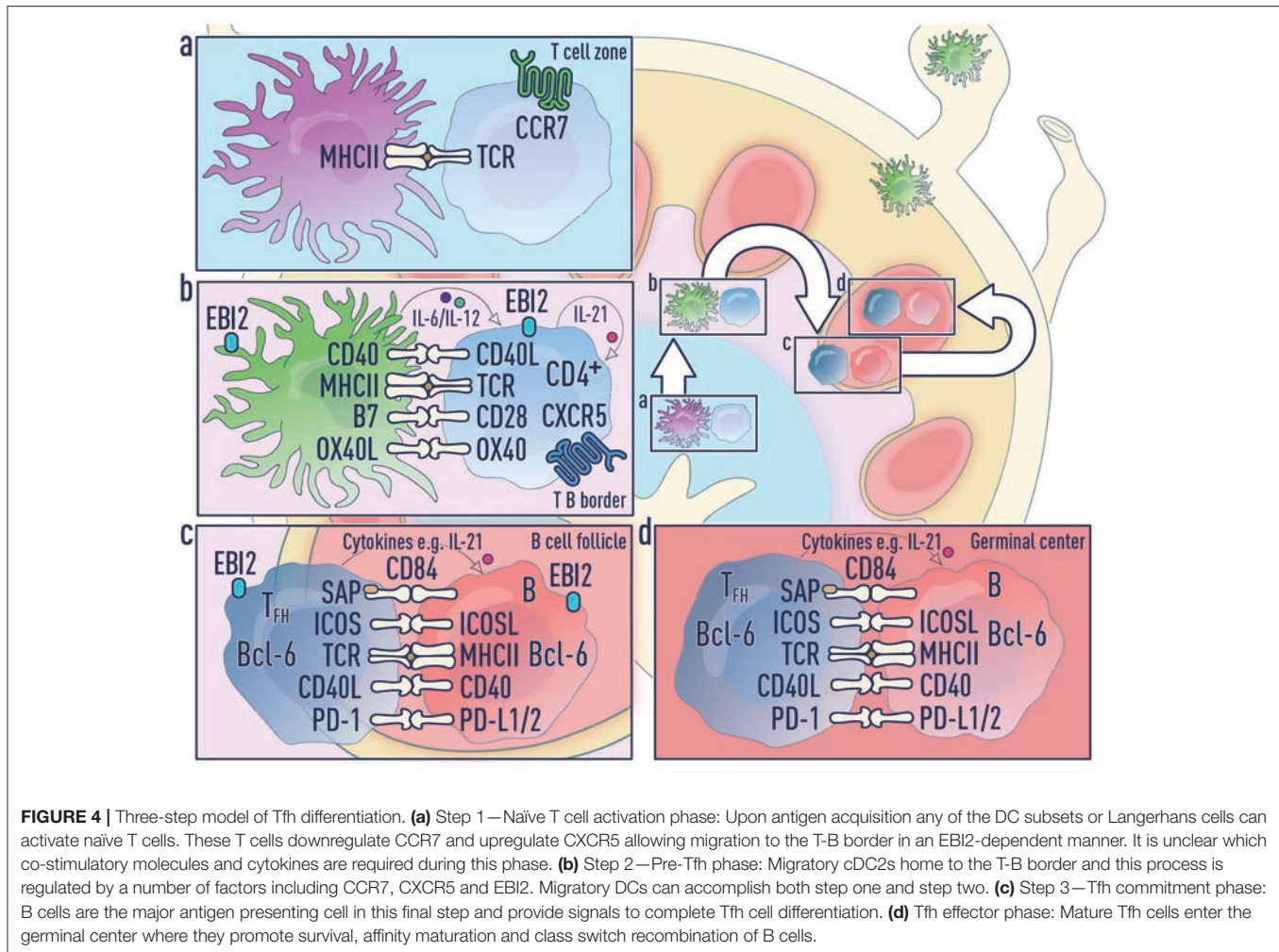
FIGURE 3 | Antigen availability and APC dependency. We propose that antigen availability in secondary lymphoid organs (SLOs) determines which antigen presenting cells (APCs) are able to support Tfh priming. At very high antigen concentrations B cells can serve as the sole APC to support Tfh development. Decreasing antigen concentrations progressively increases the dependency on APCs that are more effective in priming Tfh cells. Migratory cDC2s are the most potent Tfh-priming APC and are both necessary and sufficient at low antigen concentrations.

- Any of these DC subsets upon antigen acquisition can activate naïve T cells. These T cells migrate to the T-B border in a CXCR5- and/or EBI2-dependent process.
2. **Pre-Tfh differentiation by migratory cDC2s:** Migratory cDC2s home to the T-B border and this process is regulated by a number of factors including CCR7 (106), CXCR5 (2, 30, 43) and EBI2 (48). As migratory cDC2s uniquely position themselves in the IFZ, they will efficiently support pre-Tfh differentiation. This makes cDC2s increasingly important when the level of antigen in the SLO is limiting. In the absence of free draining antigen, migratory DCs can accomplish both step one and step two.
 3. **Tfh commitment by B cells:** As has been extensively described, B cells become the major antigen presenting cell in the final stage and provide signals including ICOSL/ICOS, CD40/CD40L, and CD84/CD84-SAP to complete Tfh cell differentiation (1, 2, 63).

SUMMARY AND FUTURE DIRECTIONS

DCs are heterogeneous, and multiple subsets have been implicated in priming Tfh cells. Although our three-step model incorporates these findings, it is important to bear in mind that there is currently no definitive evidence clarifying the individual role of each of these DC subsets in Tfh cell differentiation. It is likely that the type and body location of immune insult determines the DC subsets responsible. The biggest hurdle toward addressing this is the ability to specifically deplete either resident or migratory cDC subsets *in vivo*. Identifying factors that uniquely drive the development, maturation, or migration of either resident or migratory cDC1s and cDC2s would be critical for the generation of such murine models.

While many Tfh cell differentiation factors including cytokines and co-stimulatory molecules have been described, it still remains unclear whether DCs are indeed the primary



source of these factors. If so, which DC subsets provide them, at what stage during Tfh differentiation are these produced, where within the SLO are they secreted, and is this in conjunction with antigen presentation? Depletion of these factors within specific DC subsets would be one approach toward addressing these questions. Further, given that stromal cells and DCs appear to secrete some of the same factors that drive Tfh cell differentiation (58, 62), it would be critical to delineate if these cell types, which may occupy the same niches within the SLOs, act in concert to efficiently induce humoral responses.

While the migration of T cells from the T cell zone to the T-B border, and then to the follicle during Tfh differentiation, is well studied, there remains much to be understood about the spatio-temporal dynamics of this process with respect to T cell interactions with specific DC subsets. In other words, during the DC-phase of Tfh cell differentiation, do the differentiating T cells sequentially encounter different DC subsets? What specific signaling pathways and transcriptional programs are activated at each step of this process that then drives the next step of Tfh cell differentiation? Development of advanced

microscopic techniques like multi-photon microscopy and multi-parameter immunofluorescence to simultaneously visualize T cells and antigen-bearing DC subsets would be required to address these questions. These techniques could be used in conjunction with next generation sequencing platforms such as single-cell RNA-Seq and spatial transcriptomics to identify the impact of different DC subsets on specific stages of Tfh cell differentiation.

Finally, Tfh cells can play either a beneficial or detrimental role in different diseases. First, Tfh cells are crucial in mediating protective antibody responses against pathogens as well as driving effective vaccine responses. However, we suggest that current vaccination strategies may be suboptimal in reaching DCs that are most efficient in priming Tfh responses (30). On the other hand, Tfh cells have been implicated in a diverse range of diseases such as allergy, autoimmunity, transplant rejection, and even cancer (107–112). Currently, little is known regarding the DC-Tfh axis in the context of disease. Work will be needed to determine how DCs impact disease initiation, progression, or severity by controlling the magnitude and/or type of Tfh response. Manipulation of DCs could potentially provide a

therapeutic avenue to correct “misguided” or inadequate Tfh responses.

AUTHOR CONTRIBUTIONS

JK, SE, and AW wrote the first draft of the manuscript. SA and UY wrote sections of the manuscript and contributed intellectual review. All authors contributed to manuscript revision, read, and approved the submitted version.

REFERENCES

- Crotty S. T follicular helper cell differentiation, function, and roles in disease. *Immunity* (2014) 41:529–42. doi: 10.1016/j.immuni.2014.10.004
- Ballesteros-Tato A, Randall TD. Priming of T follicular helper cells by dendritic cells. *Immunol Cell Biol*. (2014) 92:22–7. doi: 10.1038/icb.2013.62
- Qi H. T follicular helper cells in space-time. *Nat Rev Immunol*. (2016) 16:612–25. doi: 10.1038/nri.2016.94
- Tangye SG, Ma CS, Brink R, Deenick EK. The good, the bad and the ugly - TFH cells in human health and disease. *Nat Rev Immunol*. (2013) 13:412–26. doi: 10.1038/nri3447
- Schmitt N, Morita R, Bourdery L, Bentebibel SE, Zurawski SM, Banchereau J, et al. Human dendritic cells induce the differentiation of interleukin-21-producing T follicular helper-like cells through interleukin-12. *Immunity* (2009) 31:158–69. doi: 10.1016/j.immuni.2009.04.016
- Goenka R, Barnett LG, Silver JS, O'Neill PJ, Hunter CA, Cancro MP, et al. Cutting edge: dendritic cell-restricted antigen presentation initiates the follicular helper T cell program but cannot complete ultimate effector differentiation. *J Immunol*. (2011) 187:1091–5. doi: 10.4049/jimmunol.1100853
- Benson RA, MacLeod MK, Hale BG, Patakas A, Garside P, Brewer JM. Antigen presentation kinetics control T cell/dendritic cell interactions and follicular helper T cell generation *in vivo*. *eLife* (2015) 4:e06994. doi: 10.7554/eLife.06994
- Klechevsky E, Morita R, Liu M, Cao Y, Coquery S, Thompson-Snipes L, et al. Functional specializations of human epidermal Langerhans cells and CD14+ dermal dendritic cells. *Immunity* (2008) 29:497–510. doi: 10.1016/j.immuni.2008.07.013
- Baumjohann D, Okada T, Ansel KM. Cutting Edge: Distinct waves of BCL6 expression during T follicular helper cell development. *J Immunol*. (2011) 187:2089–92. doi: 10.4049/jimmunol.1101393
- Poholek AC, Hansen K, Hernandez SG, Eto D, Chande A, Weinstein JS, et al. *In vivo* regulation of Bcl6 and T follicular helper cell development. *J Immunol*. (2010) 185:313–26. doi: 10.4049/jimmunol.0904023
- Baumjohann D, Preite S, Reboldi A, Ronchi F, Ansel KM, Lanzavecchia A, et al. Persistent antigen and germinal center B cells sustain T follicular helper cell responses and phenotype. *Immunity* (2013) 38:596–605. doi: 10.1016/j.immuni.2012.11.020
- Dahlgren MW, Gustafsson-Hedberg T, Livingston M, Cucak H, Alsen S, Yrlid U, et al. T follicular helper, but not Th1, cell differentiation in the absence of conventional dendritic cells. *J Immunol*. (2015) 194:5187–99. doi: 10.4049/jimmunol.1401938
- Reinhardt RL, Liang HE, Locksley RM. Cytokine-secreting follicular T cells shape the antibody repertoire. *Nat Immunol*. (2009) 10:385–93. doi: 10.1038/ni.1715
- Morita R, Schmitt N, Bentebibel SE, Ranganathan R, Bourdery L, Zurawski G, et al. Human blood CXCR5+ CD4+ T cells are counterparts of T follicular cells and contain specific subsets that differentially support antibody secretion. *Immunity* (2011) 34:108–21. doi: 10.1016/j.immuni.2010.12.012
- Pattarini L, Trichot C, Bogiatzi S, Grandclaudon M, Meller S, Keuylia Z, Durand M, et al. TSLP-activated dendritic cells induce human T follicular helper cell differentiation through OX40-ligand. *J Exp Med*. (2017) 214:1529–46. doi: 10.1084/jem.20150402

FUNDING

This work was supported by R01 AI108829 (SE) and CTSA UL1 TR001863 (SE), R21 AI133440 (AW), R21 AI135221 (AW).

ACKNOWLEDGMENTS

We would like to thank Matt Wimsatt for generating figures, and Iiro Taneli Helenius for critical review of the manuscript.

- Meredith MM, Liu K, Darrasse-Jeze G, Kamphorst AO, Schreiber HA, Guermontez P, et al. Expression of the zinc finger transcription factor zDC (Zbtb46, Btd4) defines the classical dendritic cell lineage. *J Exp Med*. (2012) 209:1153–65. doi: 10.1084/jem.20112675
- Satpathy AT, Kc W, Albring JC, Edelson BT, Kretzer NM, Bhattacharya D, et al. Zbtb46 expression distinguishes classical dendritic cells and their committed progenitors from other immune lineages. *J Exp Med*. (2012) 209:1135–52. doi: 10.1084/jem.20120030
- Durai V, Murphy KM. Functions of murine dendritic cells. *Immunity* (2016) 45:719–36. doi: 10.1016/j.immuni.2016.10.010
- Heath WR, Carbone FR. The skin-resident and migratory immune system in steady state and memory: innate lymphocytes, dendritic cells and T cells. *Nat Immunol*. (2013) 14:978–85. doi: 10.1038/ni.2680
- Dudziak D, Kamphorst AO, Heidkamp GF, Buchholz VR, Trumpfheller C, Yamazaki S, et al. Differential antigen processing by dendritic cell subsets *in vivo*. *Science* (2007) 315:107–11. doi: 10.1126/science.1136080
- Pulendran B, Smith JL, Caspary G, Brasel K, Pettit D, Maraskovsky E, et al. Distinct dendritic cell subsets differentially regulate the class of immune response *in vivo*. *Proc Natl Acad Sci USA*. (1999) 96:1036–41. doi: 10.1073/pnas.96.3.1036
- Shortman K, Heath WR. The CD8+ dendritic cell subset. *Immunol Rev*. (2010) 234:18–31. doi: 10.1111/j.0105-2896.2009.00870.x
- Satpathy AT, Wu X, Albring JC, Murphy KM. Re(de)fining the dendritic cell lineage. *Nat Immunol*. (2012) 13:1145–54. doi: 10.1038/ni.2467
- Geissmann F, Jung S, Littman DR. Blood monocytes consist of two principal subsets with distinct migratory properties. *Immunity* (2003) 19:71–82. doi: 10.1016/S1074-7613(03)00174-2
- Kashem SW, Haniffa M, Kaplan DH. Antigen-presenting cells in the skin. *Annu Rev Immunol*. (2017) 35:469–99. doi: 10.1146/annurev-immunol-051116-052215
- Itano AA, McSorley SJ, Reinhardt RL, Ehst BD, Ingulli E, Rudensky AY, et al. Distinct dendritic cell populations sequentially present antigen to CD4 T cells and stimulate different aspects of cell-mediated immunity. *Immunity* (2003) 19:47–57. doi: 10.1016/S1074-7613(03)00175-4
- Gerner MY, Torabi-Parizi P, Germain RN. Strategically localized dendritic cells promote rapid T cell responses to lymph-borne particulate antigens. *Immunity* (2015) 42:172–85. doi: 10.1016/j.immuni.2014.12.024
- Tozuka M, Oka T, Jounai N, Egawa G, Ishii KJ, Kabashima K, et al. Efficient antigen delivery to the draining lymph nodes is a key component in the immunogenic pathway of the intradermal vaccine. *J Dermatol Sci*. (2016) 82:38–45. doi: 10.1016/j.jdermsci.2015.11.008
- Woodruff MC, Heesters BA, Herndon CN, Groom JR, Thomas PG, Luster AD, et al. Trans-nodal migration of resident dendritic cells into medullary interfollicular regions initiates immunity to influenza vaccine. *J Exp Med*. (2014) 211:1611–21. doi: 10.1084/jem.20132327
- Krishnaswamy JK, Gowthaman U, Zhang B, Mattsson J, Szeponik L, Liu D, et al. Migratory CD11b+ conventional dendritic cells induce T follicular helper cell-dependent antibody responses. *Sci Immunol*. (2017) 2:eam9169. doi: 10.1126/sciimmunol.aam9169
- Levin C, Bonduelle O, Nuttens C, Primard C, Verrier B, Boissonnas A, et al. Critical role for skin-derived migratory DCs and langerhans cells in TFH and GC responses after intradermal immunization. *J Invest Dermatol*. (2017) 137:1905–13. doi: 10.1016/j.jid.2017.04.016

32. Gonzalez SF, Lukacs-Kornek V, Kuligowski MP, Pitcher LA, Degn SE, Kim YA, et al. Capture of influenza by medullary dendritic cells via SIGN-R1 is essential for humoral immunity in draining lymph nodes. *Nat Immunol.* (2010) 11:427–34. doi: 10.1038/ni.1856
33. Miyauchi K, Sugimoto-Ishige A, Harada Y, Adachi Y, Usami Y, Kaji T, et al. Protective neutralizing influenza antibody response in the absence of T follicular helper cells. *Nat Immunol.* (2016) 17:1447–58. doi: 10.1038/ni.3563
34. Calabro S, Gallman A, Gowthaman U, Liu D, Chen P, Liu J, et al. Bridging channel dendritic cells induce immunity to transfused red blood cells. *J Exp Med.* (2016) 213:887–96. doi: 10.1084/jem.20151720
35. Li J, Lu E, Yi T, Cyster JG. EBI2 augments Tfh cell fate by promoting interaction with IL-2-quenching dendritic cells. *Nature* (2016) 533:110–4. doi: 10.1038/nature17947
36. Yao C, Zurawski SM, Jarrett ES, Chicoine B, Crabtree J, Peterson EJ, et al. Skin dendritic cells induce follicular helper T cells and protective humoral immune responses. *J Allergy Clin Immunol.* (2015) 136:1387–97.e1–7. doi: 10.1016/j.jaci.2015.04.001
37. Kato Y, Zaid A, Davey GM, Mueller SN, Nutt SL, Zotos D, et al. Targeting antigen to clec9a primes follicular th cell memory responses capable of robust recall. *J Immunol.* (2015) 195:1006–14. doi: 10.4049/jimmunol.1500767
38. Lahoud MH, Ahmet F, Kitsoulis S, Wan SS, Vremec D, Lee CN, et al. Targeting antigen to mouse dendritic cells via Clec9A induces potent CD4 T cell responses biased toward a follicular helper phenotype. *J Immunol.* (2011) 187:842–50. doi: 10.4049/jimmunol.1101176
39. Shin C, Han JA, Koh H, Choi B, Cho Y, Jeong H, et al. CD8alpha(–) Dendritic cells induce antigen-specific t follicular helper cells generating efficient humoral immune responses. *Cell Rep.* (2015) 11:1929–40. doi: 10.1016/j.celrep.2015.05.042
40. Kumamoto Y, Hirai T, Wong PW, Kaplan DH, Iwasaki A. CD301b+ dendritic cells suppress T follicular helper cells and antibody responses to protein antigens. *eLife* (2016) 5:e17979. doi: 10.7554/eLife.17979
41. Ingulli E, Ulman DR, Lucido MM, Jenkins MK. In situ analysis reveals physical interactions between CD11b+ dendritic cells and antigen-specific CD4 T cells after subcutaneous injection of antigen. *J Immunol.* (2002) 169:2247–52. doi: 10.4049/jimmunol.169.5.2247
42. Schlitzer A, McGovern N, Teo P, Zelante T, Atarashi K, Low D, et al. IRF4 transcription factor-dependent CD11b+ dendritic cells in human and mouse control mucosal IL-17 cytokine responses. *Immunity* (2013) 38:970–83. doi: 10.1016/j.immuni.2013.04.011
43. Leon B, Ballesteros-Tato A, Browning JL, Dunn R, Randall TD, Lund FE. Regulation of T(H)2 development by CXCR5+ dendritic cells and lymphotoxin-expressing B cells. *Nat Immunol.* (2012) 13:681–90. doi: 10.1038/ni.2309
44. Kerfoot SM, Yaari G, Patel JR, Johnson KL, Gonzalez DG, Kleinstein SH, et al. Germinal center B cell and T follicular helper cell development initiates in the interfollicular zone. *Immunity* (2011) 34:947–60. doi: 10.1016/j.immuni.2011.03.024
45. Gerner MY, Kastenmuller W, Ifrim I, Kabat J, Germain RN. Histo-cytometry: a method for highly multiplex quantitative tissue imaging analysis applied to dendritic cell subset microanatomy in lymph nodes. *Immunity* (2012) 37:364–76. doi: 10.1016/j.immuni.2012.07.011
46. Calabro S, Liu D, Gallman A, Nascimento MS, Yu Z, Zhang TT, et al. Differential intrasplenic migration of dendritic cell subsets tailors adaptive immunity. *Cell Rep.* (2016) 16:2472–85. doi: 10.1016/j.celrep.2016.07.076
47. Shin C, Han JA, Choi B, Cho YK, Do Y, Ryu S. Intrinsic features of the CD8alpha(–) dendritic cell subset in inducing functional T follicular helper cells. *Immunol Lett.* (2016) 172:21–8. doi: 10.1016/j.imlet.2016.01.009
48. Yi T, Cyster JG. EBI2-mediated bridging channel positioning supports splenic dendritic cell homeostasis and particulate antigen capture. *eLife* (2013) 2:e00757. doi: 10.7554/eLife.00757
49. Gatto D, Wood K, Caminschi I, Murphy-Durland D, Schofield P, Christ D, et al. The chemotactic receptor EBI2 regulates the homeostasis, localization and immunological function of splenic dendritic cells. *Nat Immunol.* (2013) 14:446–53. doi: 10.1038/ni.2555
50. Krishnaswamy JK, Singh A, Gowthaman U, Wu R, Gorrepati P, Sales Nascimento M, et al. Coincidental loss of DOCK8 function in NLRP10-deficient and C3H/HeJ mice results in defective dendritic cell migration. *Proc Natl Acad Sci USA.* (2015) 112:3056–61. doi: 10.1073/pnas.1501554112
51. Barbet G, Sander LE, Geswell M, Leonardi I, Cerutti A, Iliev I, et al. Sensing microbial viability through bacterial rna augments t follicular helper cell and antibody responses. *Immunity* (2018) 48:584–598 e5. doi: 10.1016/j.immuni.2018.02.015
52. Chakarov S, Fazilleau N. Monocyte-derived dendritic cells promote T follicular helper cell differentiation. *EMBO Mol Med.* (2014) 6:590–603. doi: 10.1002/emmm.201403841
53. Zimara N, Florian C, Schmid M, Malissen B, Kissenpfennig A, Mannel DN, et al. Langerhans cells promote early germinal center formation in response to Leishmania-derived cutaneous antigens. *European J Immunol.* (2014) 44:2955–67. doi: 10.1002/eji.201344263
54. Kissenpfennig A, Henri S, Dubois B, Laplace-Builhe C, Perrin P, Romani N, et al. Dynamics and function of Langerhans cells in vivo: dermal dendritic cells colonize lymph node areas distinct from slower migrating Langerhans cells. *Immunity* (2005) 22:643–54. doi: 10.1016/j.immuni.2005.04.004
55. Tomura M, Hata A, Matsuoka S, Shand FH, Nakanishi Y, Ikebuchi R, et al. Tracking and quantification of dendritic cell migration and antigen trafficking between the skin and lymph nodes. *Sci Rep.* (2014) 4:6030. doi: 10.1038/srep06030
56. Worbs T, Hammerschmidt SI, Forster R. Dendritic cell migration in health and disease. *Nat Rev Immunol.* (2017) 17:30–48. doi: 10.1038/nri.2016.116
57. Krishnaswamy JK, Chu, TC, Eisenbarth SC. Beyond pattern recognition: NOD-like receptors in dendritic cells. *Trends Immunol.* (2013) 34:224–33. doi: 10.1016/j.it.2012.12.003
58. Cucak H, Yrlid U, Reizis B, Kalinke U, Johansson-Lindbom B. Type I interferon signaling in dendritic cells stimulates the development of lymph-node-resident T follicular helper cells. *Immunity* (2009) 31:491–501. doi: 10.1016/j.immuni.2009.07.005
59. Ugolini M, Gerhard J, Burkert S, Jensen KJ, Georg P, Ebner F, et al. Recognition of microbial viability via TLR8 drives TFH cell differentiation and vaccine responses. *Nat Immunol.* (2018) 19:386–96. doi: 10.1038/s41590-018-0068-4
60. Eisenbarth SC, Colegio OR, O'Connor W, Sutterwala FS, Flavell RA. Crucial role for the Nalp3 inflammasome in the immunostimulatory properties of aluminium adjuvants. *Nature* (2008) 453:1122–6. doi: 10.1038/nature06939
61. Li H, Willingham SB, Ting JP, Re F. Cutting edge: inflammasome activation by alum and alum's adjuvant effect are mediated by NLRP3. *J Immunol.* (2008) 181:17–21. doi: 10.4049/jimmunol.181.1.17
62. Chen X, Ma W, Zhang T, Wu L, Qi H. Phenotypic Tfh development promoted by CXCR5-controlled re-localization and IL-6 from radiation-resistant cells. *Protein Cell* (2015) 6:825–32. doi: 10.1007/s13238-015-0210-0
63. Deenick EK, Ma CS, Brink R, Tangye SG. Regulation of T follicular helper cell formation and function by antigen presenting cells. *Curr Opin Immun.* (2011) 23:111–8. doi: 10.1016/j.coi.2010.10.007
64. Kulkarni RR, Rasheed MA, Bhaumik SK, Ranjan P, Cao W, Davis C, et al. Activation of the RIG-I pathway during influenza vaccination enhances the germinal center reaction, promotes T follicular helper cell induction, provides a dose-sparing effect and protective immunity. *J Virol.* (2014) 88:13990–4001. doi: 10.1128/JVI.02273-14
65. Tubo NJ, Jenkins MK. TCR signal quantity and quality in CD4(+) T cell differentiation. *Trends Immunol.* (2014) 35:591–6. doi: 10.1016/j.it.2014.09.008
66. Fazilleau N, McHeyzer-Williams LJ, Rosen H, McHeyzer-Williams MG. The function of follicular helper T cells is regulated by the strength of T cell antigen receptor binding. *Nat Immunol.* (2009) 10:375–84. doi: 10.1038/ni.1704
67. Tam HH, Melo MB, Kang M, Pelet JM, Ruda VM, Foley MH, et al. Sustained antigen availability during germinal center initiation enhances antibody responses to vaccination. *Proc Natl Acad Sci USA.* (2016) 113:E6639–48. doi: 10.1073/pnas.1606050113
68. Ploquin MJ, Eksmond U, Kassiotis G. B cells and TCR avidity determine distinct functions of CD4+ T cells in retroviral infection. *J Immunol.* (2011) 187:3321–30. doi: 10.4049/jimmunol.1101006
69. Tubo NJ, Pagan AJ, Taylor JJ, Nelson RW, Linehan JL, Ertelt JM, et al. Single naive CD4+ T cells from a diverse repertoire produce different effector cell types during infection. *Cell* (2013) 153:785–96. doi: 10.1016/j.cell.2013.04.007

70. Vander Lugt B, Khan AA, Hackney JA, Agrawal S, Lesch J, Zhou M, et al. Transcriptional programming of dendritic cells for enhanced MHC class II antigen presentation. *Nat Immunol.* (2014) 15:161–7. doi: 10.1038/ni.2795
71. Shahinian A, Pfeffer K, Lee KP, Kundig TM, Kishihara K, Wakeham A, et al. Differential T cell costimulatory requirements in CD28-deficient mice. *Science* (1993) 261:609–12. doi: 10.1126/science.7688139
72. Lane P, Burdet C, Hubele S, Scheidegger D, Muller U, McConnell F, et al. B cell function in mice transgenic for mCTLA4-H gamma 1: lack of germinal centers correlated with poor affinity maturation and class switching despite normal priming of CD4+ T cells. *J Exp Med.* (1994) 179:819–30. doi: 10.1084/jem.179.3.819
73. Flynn S, Toellner KM, Raykundalia C, Goodall M, Lane P. CD4 T cell cytokine differentiation: the B cell activation molecule, OX40 ligand, instructs CD4 T cells to express interleukin 4 and upregulates expression of the chemokine receptor, Blr-1. *J Exp Med.* (1998) 188:297–304. doi: 10.1084/jem.188.2.297
74. Walker LS, Gulbranson-Judge A, Flynn S, Brocker T, Raykundalia C, Goodall M, et al. Compromised OX40 function in CD28-deficient mice is linked with failure to develop CXCL12 chemokine receptor 5-positive CD4 cells and germinal centers. *J Exp Med.* (1999) 190:1115–22. doi: 10.1084/jem.190.8.1115
75. Watanabe M, Fujihara C, Radtke AJ, Chiang YJ, Bhatia S, Germain RN, et al. Co-stimulatory function in primary germinal center responses: CD40 and B7 are required on distinct antigen-presenting cells. *J Exp Med.* (2017) 214:2795–810. doi: 10.1084/jem.20161955
76. Akiba H, Takeda K, Kojima Y, Usui Y, Harada N, Yamazaki T, et al. The role of ICOS in the CXCR5+ follicular B helper T cell maintenance *in vivo*. *J Immunol.* (2005) 175:2340–8. doi: 10.4049/jimmunol.175.4.2340
77. Bossaller L, Burger J, Draeger R, Grimbacher B, Knoth R, Plebani A, et al. ICOS deficiency is associated with a severe reduction of CXCR5+CD4 germinal center Th cells. *J Immunol.* (2006) 177:4927–32. doi: 10.4049/jimmunol.177.7.4927
78. Brocker T, Gulbranson-Judge A, Flynn S, Riedinger M, Raykundalia C, Lane P. CD4 T cell traffic control: *in vivo* evidence that ligation of OX40 on CD4 T cells by OX40-ligand expressed on dendritic cells leads to the accumulation of CD4 T cells in B follicles. *Eur J Immunol.* (1999) 29:1610–6.
79. Ohshima Y, Tanaka Y, Tozawa H, Takahashi Y, Maliszewski C, Delespesse G. Expression and function of OX40 ligand on human dendritic cells. *J Immunol.* (1997) 159:3838–48.
80. Fillatreau S, Gray D. T cell accumulation in B cell follicles is regulated by dendritic cells and is independent of B cell activation. *J Exp Med.* (2003) 197:195–206. doi: 10.1084/jem.20021750
81. Boettler T, Choi YS, Salek-Ardakani S, Cheng Y, Moeckel F, Croft M, et al. Exogenous OX40 stimulation during lymphocytic choriomeningitis virus infection impairs follicular Th cell differentiation and diverts CD4 T cells into the effector lineage by upregulating Blimp-1. *J Immunol.* (2013) 191:5026–35. doi: 10.4049/jimmunol.1300013
82. Linterman MA, Denton AE, Divekar DP, Zvetkova I, Kane L, Ferreira M, et al. CD28 expression is required after T cell priming for helper T cell responses and protective immunity to infection. *eLife* (2014) 3:e03180. doi: 10.7554/eLife.03180
83. Tahiliani V, Hutchinson TE, Abboud G, Croft M, Salek-Ardakani S. OX40 Cooperates with ICOS To amplify Follicular Th cell development and germinal center reactions during infection. *J Immunol.* (2017) 198:218–28. doi: 10.4049/jimmunol.1601356
84. Pratama A, Ramiscal RR, Silva DG, Das SK, Athanasopoulos V, Fitch J, et al. Roquin-2 shares functions with its paralog Roquin-1 in the repression of mRNAs controlling T follicular helper cells and systemic inflammation. *Immunity* (2013) 38:669–80. doi: 10.1016/j.immuni.2013.01.011
85. Vogel KU, Edelmann SL, Jeltsch KM, Bertossi A, Heger K, Heinz GA, et al. Roquin paralogs 1 and 2 redundantly repress the Icos and OX40 costimulator mRNAs and control follicular helper T cell differentiation. *Immunity* (2013) 38:655–68. doi: 10.1016/j.immuni.2012.12.004
86. Pratama A, Srivastava M, Williams NJ, Papa I, Lee SK, Dinh XT, et al. MicroRNA-146a regulates ICOS-ICOSL signalling to limit accumulation of T follicular helper cells and germinal centres. *Nat Commun.* (2015) 6:6436. doi: 10.1038/ncomms7436
87. Nurieva RI, Chung Y, Hwang D, Yang XO, Kang HS, Ma L, et al. Generation of T follicular helper cells is mediated by interleukin-21 but independent of T helper 1, 2, or 17 cell lineages. *Immunity* (2008) 29:138–49. doi: 10.1016/j.immuni.2008.05.009
88. Choi YS, Kageyama R, Eto D, Escobar TC, Johnston RJ, Monticelli L, et al. ICOS receptor instructs T follicular helper cell versus effector cell differentiation via induction of the transcriptional repressor Bcl6. *Immunity* (2011) 34:932–46. doi: 10.1016/j.immuni.2011.03.023
89. Auderset F, Schuster S, Fasnacht N, Coutaz M, Charmoy M, Koch U, et al. Notch signaling regulates follicular helper T cell differentiation. *J Immunol.* (2013) 191:2344–50. doi: 10.4049/jimmunol.1300643
90. Dell'Aquila M, Reinhardt RL. Notch signaling represents an important checkpoint between follicular T-helper and canonical T-helper 2 cell fate. *Mucosal Immunol.* (2018) 11:1079–91. doi: 10.1038/s41385-018-0012-9
91. Choi YS, Eto D, Yang JA, Lao C, Crotty S. Cutting edge: STAT1 is required for IL-6-mediated Bcl6 induction for early follicular helper cell differentiation. *J Immunol.* (2013) 190:3049–53. doi: 10.4049/jimmunol.1203032
92. Nurieva RI, Chung Y, Martinez GJ, Yang XO, Tanaka S, Matskevitch TD, et al. Bcl6 mediates the development of T follicular helper cells. *Science* (2009) 325:1001–5. doi: 10.1126/science.1176676
93. Eddahri F, Denanglaire S, Bureau F, Spolski R, Leonard WJ, Leo O, et al. Interleukin-6/STAT3 signaling regulates the ability of naive T cells to acquire B-cell help capacities. *Blood* (2009) 113:2426–33. doi: 10.1182/blood-2008-04-154682
94. Kim SJ, Zou YR, Goldstein J, Reizis B, Diamond B. Tolerogenic function of Blimp-1 in dendritic cells. *J Exp Med.* (2011) 208:2193–9. doi: 10.1084/jem.20110658
95. Kim SJ, Schatzle S, Ahmed SS, Haap W, Jang SH, Gregersen PK, et al. Increased cathepsin S in Prdm1^{-/-} dendritic cells alters the TFH cell repertoire and contributes to lupus. *Nat Immunol.* (2017) 18:1016–24. doi: 10.1038/ni.3793
96. Hercor M, Anciaux M, Denanglaire S, Debuissson D, Leo O, Andris F. Antigen-presenting cell-derived IL-6 restricts the expression of GATA3 and IL-4 by follicular helper T cells. *J Leukoc Biol.* (2017) 101:5–14. doi: 10.1189/jlb.1HI115-511R
97. Ma CS, Suryani S, Avery DT, Chan A, Nanan R, Santner-Nanan B, et al. Early commitment of naive human CD4⁺ T cells to the T follicular helper (TFH) cell lineage is induced by IL-12. *Immunol Cell Biol.* (2009) 87:590–600. doi: 10.1038/icb.2009.64
98. Leal Rojas IM, Mok WH, Pearson FE, Minoda Y, Kenna TJ, Barnard RT, et al. Human Blood CD1c⁺ Dendritic Cells Promote Th1 and Th17 Effector Function in Memory CD4⁺ T Cells. *Front Immunol.* (2017) 8:971. doi: 10.3389/fimmu.2017.00971
99. Collin M, McGovern N, Haniffa M. Human dendritic cell subsets. *Immunology* (2013) 140:22–30. doi: 10.1111/imm.12117
100. Maldonado-Lopez R, De Smedt T, Michel P, Godfroid J, Pajak B, Heirman C, et al. CD8a+ and CD8a- subclasses of dendritic cells direct the development of distinct T helper cells *in vivo*. *J Exp Med.* (1999) 189:587–92. doi: 10.1084/jem.189.3.587
101. Gringhuis SI, Kaptein TM, Wevers BA, van der Vlist M, Klaver EJ, van Die I, et al. Fucose-based PAMPs prime dendritic cells for follicular T helper cell polarization via DC-SIGN-dependent IL-27 production. *Nat Commun.* (2014) 5:5074. doi: 10.1038/ncomms6074
102. Batten M, Ramamoorthi N, Kljavin NM, Ma CS, Cox JH, Dengler HS, et al. IL-27 supports germinal center function by enhancing IL-21 production and the function of T follicular helper cells. *J Exp Med.* (2010) 207:2895–906. doi: 10.1084/jem.20100064
103. Ray JP, Staron MM, Shyer JA, Ho PC, Marshall HD, Gray SM, et al. The Interleukin-2-mTORC1 kinase axis defines the signaling, differentiation, and metabolism of T Helper 1 and Follicular B Helper T Cells. *Immunity* (2015) 43:690–702. doi: 10.1016/j.immuni.2015.08.017
104. Waldmann TA, Goldman CK, Robb RJ, Depper JM, Leonard WJ, Sharrow SO, et al. Expression of interleukin 2 receptors on activated human B cells. *J Exp Med.* (1984) 160:1450–66. doi: 10.1084/jem.160.5.1450
105. Haynes NM, Allen CD, Lesley R, Ansel KM, Killeen N, Cyster JG. Role of CXCR5 and CCR7 in follicular Th cell positioning and appearance of a programmed cell death gene-1-high germinal

- center-associated subpopulation. *J Immunol.* (2007) 179:5099–108. doi: 10.4049/jimmunol.179.8.5099
106. Ohl L, Mohaupt M, Czeloth N, Hintzen G, Kiafard Z, Zwirner J, et al. CCR7 governs skin dendritic cell migration under inflammatory and steady-state conditions. *Immunity* (2004) 21:279–88. doi: 10.1016/j.immuni.2004.06.014
 107. Ueno H. T follicular helper cells in human autoimmunity. *Curr Opin Immunol.* (2016) 43:24–31. doi: 10.1016/j.coi.2016.08.003
 108. Yan L, de Leur K, Hendriks RW, van der Laan LJW, Shi Y, Wang L, et al. T Follicular Helper Cells as a new target for immunosuppressive therapies. *Front Immunol.* (2017) 8:1510. doi: 10.3389/fimmu.2017.01510
 109. Schulten V, Tripple V, Seumois G, Qian Y, Scheuermann RH, Fu Z, et al. Allergen-specific immunotherapy modulates the balance of circulating Tfh and Tfr cells. *J Allergy Clin Immunol.* (2018) 141:775–777 e6. doi: 10.1016/j.jaci.2017.04.032
 110. Gong F, Qian C, Zhu H, Zhu J, Pan Y, Dong Q, et al. Circulating follicular T-helper cell subset distribution in patients with asthma. *Allergy Asthma Proc.* (2016) 37:154–61. doi: 10.2500/aap.2016.37.3982
 111. Bindea G, Mlecnik B, Tosolini M, Kirilovsky A, Waldner M, Obenauf AC, et al. Spatiotemporal dynamics of intratumoral immune cells reveal the immune landscape in human cancer. *Immunity* (2013) 39:782–95. doi: 10.1016/j.immuni.2013.10.003
 112. Gu-Trantien C, Loi S, Garaud S, Equeter C, Libin M, de Wind A, et al. CD4⁺ follicular helper T cell infiltration predicts breast cancer survival. *J Clin Invest.* (2013) 123:2873–92. doi: 10.1172/JCI67428

Conflict of Interest Statement: JK is employed by the company AstraZeneca AB.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2018 Krishnaswamy, Alsén, Yrlid, Eisenbarth and Williams. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



IL-21 Biased Alemtuzumab Induced Chronic Antibody-Mediated Rejection Is Reversed by LFA-1 Costimulation Blockade

Jean Kwun^{1*}, Jaeberm Park¹, John S. Yi², Alton B. Farris³, Allan D. Kirk¹ and Stuart J. Knechtle¹

¹ Department of Surgery, Duke Transplant Center, Duke University Medical Center, Durham, NC, United States, ² Division of Surgical Sciences, Department of Surgery, Duke University Medical Center, Durham, NC, United States, ³ Department of Pathology, Emory University School of Medicine, Atlanta, GA, United States

OPEN ACCESS

Edited by:

Georgia Fousteri,
San Raffaele Hospital (IRCCS), Italy

Reviewed by:

Carla Baan,
Erasmus University Rotterdam,
Netherlands

Christopher E. Rudd,
Université de Montréal, Canada

*Correspondence:

Jean Kwun
jean.kwun@duke.edu

Specialty section:

This article was submitted to
T Cell Biology,
a section of the journal
Frontiers in Immunology

Received: 07 May 2018

Accepted: 18 September 2018

Published: 15 October 2018

Citation:

Kwun J, Park J, Yi JS, Farris AB, Kirk AD and Knechtle SJ (2018) IL-21 Biased Alemtuzumab Induced Chronic Antibody-Mediated Rejection Is Reversed by LFA-1 Costimulation Blockade. *Front. Immunol.* 9:2323. doi: 10.3389/fimmu.2018.02323

Despite its excellent efficacy in controlling T cell mediated acute rejection, lymphocyte depletion may promote a humoral response. While T cell repopulation after depletion has been evaluated in many aspects, the B cell response has not been fully elucidated. We tested the hypothesis that the mechanisms also involve skewed T helper phenotype after lymphocytic depletion. Post-transplant immune response was measured from alemtuzumab treated hCD52Tg cardiac allograft recipients with or without anti-LFA-1 mAb. Alemtuzumab induction promoted serum DSA, allo-B cells, and CAV in humanized CD52 transgenic (hCD52Tg) mice after heterotopic heart transplantation. Additional anti-LFA-1 mAb treatment resulted in reduced DSA (Fold increase 4.75 ± 6.9 vs. 0.7 ± 0.5 ; $p < 0.01$), allo-specific B cells (0.07 ± 0.06 vs. 0.006 ± 0.002 %; $p < 0.01$), neo-intimal hyperplasia (56 ± 14 % vs. 23 ± 13 %; $p < 0.05$), arterial disease (77.8 ± 14.2 vs. 25.8 ± 20.1 %; $p < 0.05$), and fibrosis (15 ± 23.3 vs. 4.3 ± 1.65 %; $p < 0.05$) in this alemtuzumab-induced chronic antibody-mediated rejection (CAMR) model. Surprisingly, elevated serum IL-21 levels in alemtuzumab-treated mice was reduced with LFA-1 blockade. In accordance with the increased serum IL-21 level, alemtuzumab treated mice showed hyperplastic germinal center (GC) development, while the supplemental anti-LFA-1 mAb significantly reduced the GC frequency and size. We report that the incomplete T cell depletion inside of the GC leads to a systemic IL-21 dominant milieu with hyperplastic GC formation and CAMR. Conventional immunosuppression, such as tacrolimus and rapamycin, failed to reverse AMR, while co-stimulation blockade with LFA-1 corrected the GC hyperplastic response. The identification of IL-21 driven chronic AMR elucidates a novel mechanism that suggests a therapeutic approach with cytolytic induction.

Keywords: IL-21, germinal center, follicular helper T cells, heart transplantation, antibody-mediated rejection

INTRODUCTION

Long-term success of heart transplantation is limited by the development of coronary allograft vasculopathy (CAV), a hallmark of chronic rejection (CR) (1). Conventional immunosuppressive strategies, such as CNI inhibitors or rapamycin, that inhibit T cell-mediated rejection do not prevent CR; indeed, ~50% of patients develop biopsy evidence of CAV within 5 years after transplantation (2, 3). This is a particularly devastating statistic for pediatric transplant recipients because children with organ transplants have the greatest need for long-term graft survival. The inability of current T cell-directed immunosuppressive therapies to target humoral responses might explain their inability to suppress chronic rejection.

Recently, considerable progress has been made in understanding the relationships between B cells, alloantibody, and chronic rejection. Studies have demonstrated that levels of donor-specific antibodies (DSA) correlate most closely with chronic rejection (4–8). Yet, despite this demonstrated association of DSA and later graft loss, exact mechanisms underlying chronic antibody-mediated rejection (CAMR) remain unknown. In addition, a lack of satisfactory animal models further hampers progress toward understanding the mechanisms of CAMR.

Lymphocytolytic induction has been widely used in organ transplantation and autoimmune disease. Induction initiated prior to or concurrent with transplantation has been shown to be beneficial in reducing maintenance immunosuppression requirements after transplantation (9, 10), and in particular, alemtuzumab (Campath-1H) induction has been shown to be highly effective in preventing acute rejection (11). Following induction with alemtuzumab, regulatory T cells (Tregs) expand disproportionately during T cell repopulation (12). However, despite its excellent efficacy controlling T cell-mediated acute rejection, alemtuzumab may paradoxically promote alloantibody production (13, 14).

Growing evidences now show that a possible contribution of follicular helper T cells (T_{fh}) on B cell help under current immunosuppression and antibody-mediated rejection. It is also documented that agents targeting T_{fh}-B cell interaction reduced post-transplant humoral response (15, 16). While many aspects of T cell repopulation after cytolytic induction are understood, the T_{fh} and B cell response has not yet been fully elucidated. Here we report that incomplete T cell depletion inside of the germinal center (GC) leads to a systemic IL-21-dominant milieu and subsequent formation of hyperplastic GC, which results in chronic antibody-mediated rejection (CAMR). Conventional immunosuppression with tacrolimus and rapamycin failed to reverse AMR, but targeting LFA-1 corrected the GC hyperplasticity. The identification of a possible GC response and IL-21-driven CAMR elucidates a novel mechanism to understand a modern problem that suggests a therapeutic approach with cytolytic induction.

MATERIALS AND METHODS

Animal Model

Male C57BL/6 (H-2^b), 6–8 weeks of age, were purchased from The Jackson laboratory (Bar Harbor, ME). Male hCD52Tg mice (H-2^k), 6–8 weeks of age, were originally created in the Walldman lab and were a gift of Dr. Kirk, Duke University. All mice were used and maintained in accordance with the guidelines and compliance of the Emory or Duke Institutional Animal Research Ethics Committee. All animals received 10 µg alemtuzumab (Campath-1H) in 200 ml PBS i.p., on day –2, –1, +2, and +4 of transplantation to induce T cell depletion *in vivo*. Additionally, animals were either untreated or treated with 200 µg of anti-LFA-1 mAb (mCD11a, M17/4; Bioexpress) i.p., on days 0, 2, 4, and 6 (day 0 being the day of transplantation). Heart transplantation was performed using a modification of the methods described previously (17). Histopathologic analysis was performed on paraffin-embedded sections of heart allografts removed at necropsy. Sections were stained with either H and E or Elastic trichrome and were scored blindly according to the established clinical criteria for diagnosing heart transplant rejection (18, 19).

Flow Cytometry

Cell suspensions from spleens and lymph nodes were prepared by mechanical dissociation. Cell suspensions including blood was subjected to hypotonic lysis of RBCs. Isolated cells were washed in RPMI 1640 and 10% FBS and counted. The cells were then resuspended in FACS buffer (2% FBS, 0.2% Sodium azide PBS) and were stained with Biotin, PE, FITC, PerCp, Pac Orange, Pac Blue, APC, APC-Cy7, or APC conjugated antibodies directed at mouse CD3, CD4, CD8, CD19, CD25, CD38, CD4/CD8/F4/80 (Dump), FoxP3, IgD. For, allo-specific visualization, APC-Cy7-conjugated allogeneic (H-2K^b/D^b) MHC tetramer and APC-conjugated syngeneic (H-2K^k/D^k) tetramer were applied as previously described (20). MHC monomers were generated from NIH tetramer core and tetramerized with Streptavidin-APC-Cy7 and Streptavidin-APC, respectively. For T cell flow crossmatch, recipient serum samples (1:32 dilution) were incubated with C57BL/6 donor splenocytes (1 × 10⁶). Later, FITC-conjugated anti-mouse Ig was added after washing. The T cells were stained with APC-conjugated anti-CD3. Flow cytometric analysis was performed using a BD FACS LSRII or BD Forreza and analyzed using FlowJo (Tree Star, San Carlos, CA) software.

Histology, Immunohistochemistry, and Morphological Analysis

The explanted hearts underwent serial sectioning (5 µm) from the midventricular level to the base. H and E stains were performed for routine examination and grading of rejection. Elastic trichrome, B220, CD3, CD4, PNA, Ki67, and IL-21 staining was performed for morphometric analyses of arterial intimal lesions as previously reported (20). Scanned images were analyzed and measured with computer-based software (Aperio Imagescope v11). The area of grafts was quantitated by tracing the bisected explanted cardiac allografts and isografts. Luminal

(L) and intimal and luminal area (I + L) were traced and the areas quantitated. Intimal thickening was calculated according to the formula $I/I + L$ and expressed as a percentage.

Statistical Analysis

Experimental results were analyzed by a GraphPad Prism (GraphPad Software 6.0a, San Diego, CA). The log-rank test for differences in graft survival and Mann-Whitney nonparametric test were used for other data. All the data are presented as mean $\pm$ SEM unless designated in figure legend. Values of p which were < 0.05 were considered as statistically significant.

RESULTS

Chronic Antibody-Mediated Rejection After Alemtuzumab Induction

We previously reported that alemtuzumab induction prevents acute rejection in humanized CD52 transgenic (hCD52Tg) mice after heterotopic heart transplantation but promotes serum DSA, allo-B cells and CAV, making this an applicable model for studying CAMR post cytolytic induction (20). Interestingly, and as is seen clinically, this heightened humoral response was not controlled by adding either tacrolimus (**Data not shown**) or rapamycin (21). We treated humanized CD52 transgenic mice with alemtuzumab with or without anti-LFA-1 mAb and monitored DSA, allospecific B (allo-B) cells and CAV development (**Figure 1A**). We made the surprising observation that anti-LFA-1 mAb suppressed the humoral response seen in animals treated with alemtuzumab. Anti-LFA-1 mAb treatment did not change graft survival or beating quality, which remained unperturbed compared to alemtuzumab-alone treatment (**Figure 1B**). However, DSA production was greatly reduced at post-transplantation day (POD) 100 with LFA-1 blockade (**Figure 1C**). In addition, we tracked allo-B cells using MHC/Peptide tetramers (20). LFA-1 blockade resulted in significantly reduced allo-B cells in the spleen at POD 100 (**Figure 1D**). These data indicate that LFA-1 blockade prevents DSA production and suppresses allo-B cell formation, possibly by suppressing clonal B cell expansion.

LFA-1 Blockade Significantly Diminished Chronic Antibody-Mediated Rejection

Having observed a reduction in allo-B cells and DSA following anti-LFA-1 mAb treatment, we assessed the effect on CAV development. Cardiac coronary artery thickness was measured with Aperio scanscope program with elastic trichrome or Verhoeff staining. Even with significantly reduced DSA and allo-B cells after anti-LFA-1 mAb treatment, a noticeable amount of neo-intimal hyperplasia persisted, distinct from syngeneic controls (**Figure 2A**). We also noted some collapsed major coronary arteries in the anti-LFA-1 mAb-treated animals. This may represent non-DSA related CAV development. Overall, however, LFA-1 blockade significantly reduced neo-intimal hyperplasia (**Figure 2B**), diseased vessel number (**Figure 2C**), and fibrosis (**Figure 2D**) in the alemtuzumab-induced CAMR model. Over time, the non-functional heterotopic syngeneic cardiac allograft atrophied, likely due to the off-loaded left

ventricle (22) and a limited immunologic reaction. The hypotrophic condition of allografts treated with LFA-1 blockade may represent a decreased immunologic burden when compared to allografts treated with alemtuzumab alone (**Supplemental Figure 1**). Collectively, we conclude that LFA-1 blockade might prevent CAV via suppression of allo-B cells in a T cell depletion—induced CAMR model.

Systemic Cytokine Milieu During Homeostatic T Cell Repopulation

Vascular remodeling is also affected by patterns of cytokine expression. IFN- γ and other Th1 cytokines are causally implicated in stenosing vascular lesions, while Th2 cytokine expression results in abdominal aortic aneurysms (23). Many cytokines also play an important role in the control of B cell responses. IL-4 and IL-21, especially, have been shown to be important for B cell help (24, 25), and are required for an optimal humoral response (26). To address whether the decreased CAV seen during LFA-1 blockade was due to the modulation of the cytokine environment, serum IL-4, IL-21, BAFF, and IL-2 were evaluated in the absence or presence of anti-LFA-1 mAb treatment. Strikingly, IL-21 serum levels were drastically increased in alemtuzumab-treated cardiac allograft CD52Tg mouse recipients over time (**Figure 3A**). IL-4 levels were less impressively increased at POD 100 (**Figure 3B**). Serum BAFF levels were also elevated upon T cell depletion post-transplantation, similarly to what has been reported in alemtuzumab-treated human patients (27, 28). However, the serum BAFF level returned to baseline at POD 100, which might represent a transient fluctuation at an early time point, possibly due to T cell depletion—induced B cell loss (**Figure 3C**). Serum IL-2 levels were not changed over time after alemtuzumab treatment (**Figure 3D**) and suggests that only a small amount is released in the tissue. In this sense, elevated serum levels of IL-2 in CD4/CD8 mAb-treated cardiac allograft recipients might represent Th-1—driven AMR (**Supplemental Figure 2**). It is possible that the intensity of the Th1 response immediately following T cell depletion dictates later humoral responses. Interestingly, early and late IL-21 serum levels were significantly reduced to near-background levels following LFA-1 blockade at both time points (**Figures 3E,F**; $p < 0.01$). LFA-1 blockade also reduced serum IL-4 levels significantly at POD 100 (**Figure 3F**; $p < 0.05$). It is notable that IL-21 was not elevated in untreated (non-depleted) CD52Tg recipients at any time points, even with high levels of DSA and allo-B cells throughout the study course (data not shown), suggesting that in the face of an unopposed Th1 response in the absence of alemtuzumab treatment both IL-21 levels and the GC response is completely suppressed (29). Meanwhile, IL-4 was elevated in both untreated and alemtuzumab-treated recipients at POD 100. It is also notable that serum IL-21 and IL-4 levels were not completely suppressed by the addition of tacrolimus or rapamycin (**Supplemental Figure 3**). Based on the redundant yet synergistic roles of IL-4 and IL-21 in the GC response (26), insufficient suppression of either results in a robust humoral response. The segregation of IgG2a and IgG1 immunoglobulin

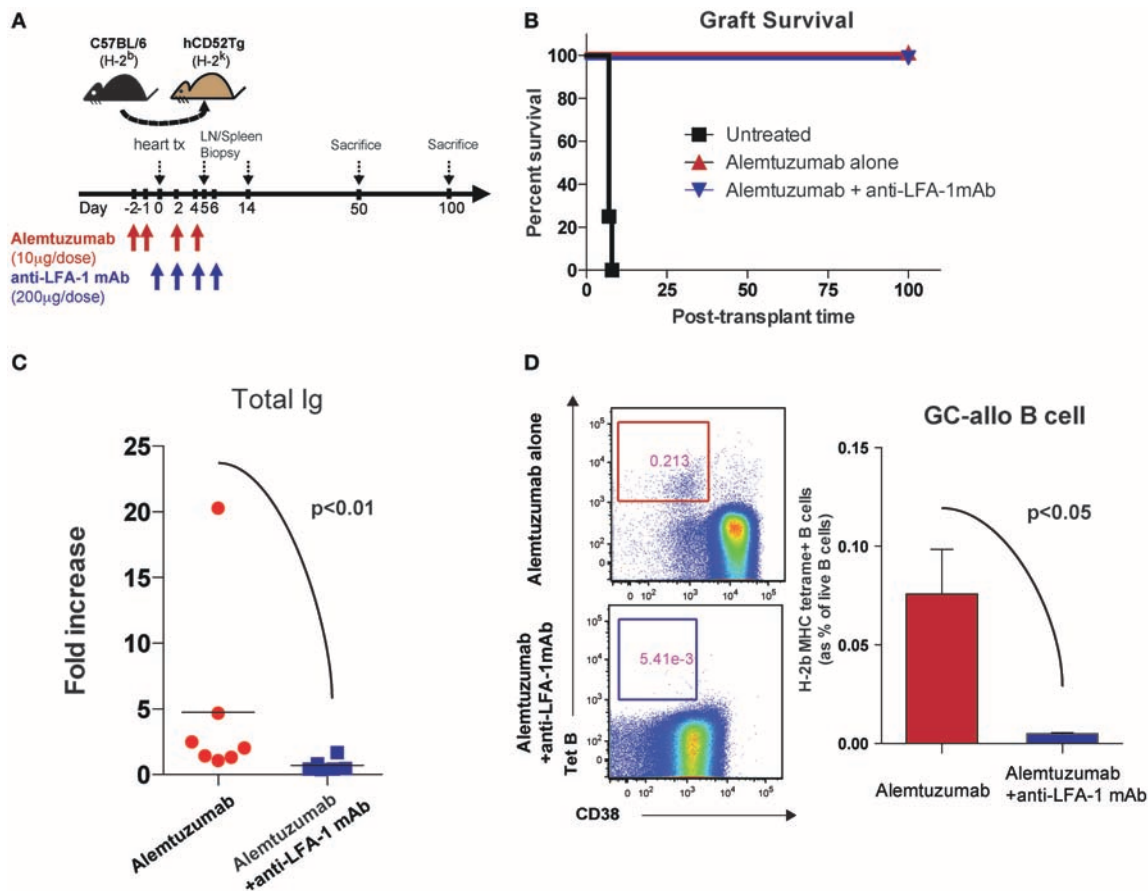


FIGURE 1 | Pre-emptive anti-LFA-1 mAb treatment reduced post-transplant DSA and allo-specific B cells in alemtuzumab treated hCD52Tg cardiac allograft recipients. **(A)** Dosing scheme and experimental design. **(B)** Graft survival of human CD52Tg mice received B6 cardiac allografts. Alemtuzumab treatment (IP, 10 μg per dose at POD -2, -1, 2, 4) with or without anti-LFA-1 mAb (KBA-1; 200 μg per dose at POD 0, 2, 4, 6) significantly prolonged graft survival (MST > 100 d) vs. untreated (MST = 9 d). **(C)** Donor-specific antibody measured by T cell flow crossmatch was significantly decreased in anti-LFA-1 treatment in Alemtuzumab induced CAMR model. **(D)** Allo-specific B cells visualized by MHC (H-2K^b/D^b) tetramer were significantly reduced with anti-LFA-1 mAb treatment from the spleen at POD100.

isotypes is often used as a marker for Th1 and Th2 responses, respectively. Concordant with the response suggested by the cytokine profile, serum from untreated animals showed both IgG1 and IgG2a dominant isotypes (possibly Th1-biased), while serum from alemtuzumab treated animals showed suppression of IgG2a isotypes (Supplemental Figure 4). Reduction of IL-21 at early and late time points with LFA-1 blockade recapitulates the reduction of DSA and allo-B cell formation shown in Figure 1. These data suggest that alemtuzumab-induced T cell depletion does not induce a Th1 response, but rather promotes an IL-21—driven humoral response, thus promoting CAMR. Furthermore, our data suggests that this response might be blocked by treatment with anti-LFA-1 mAb.

Incomplete T and GC Abrogation After Alemtuzumab Treatment

It is surprising to see rapid IL-21 production as early as 2 weeks post-T cell depletion because GC-Tfh (germinal center resident follicular helper T cells) are, theoretically, a major

source of IL-21 and IL-4 production (30). Profound T cell depletion was shown in peripheral blood and spleen, but a higher number of T cells were found in lymph nodes even 24 h after T cell depletion. We accessed GC-Tfh cells *in situ* and found evidence of an intact germinal center at 2 weeks post-transplant (Supplemental Figure 5). GC structures were also found in both lymph nodes and spleen at 24 h after full alemtuzumab doses (at POD 5; Figure 4). These data are consistent with prior human data demonstrating that even when induction results in profound peripheral T cell depletion, it does not necessarily induce complete central depletion, especially for T cells inside of GC (31). It is easily speculated that GC structure (vasculature, cell components, etc.) provides some physical protection for T cells from the T cell—depleting agent. Taken together, LFA-1 blockade suppresses an IL-21 dominant/germinal center-driven, anti-donor humoral response. IL-21 production is not unique to Tfh cells; it is also produced by other T cell lineages such as Th17, Th2, and Th1 cells (32, 33). LFA-1 is expressed on both T and B cells (34), and the initial T-B cognate interaction or later

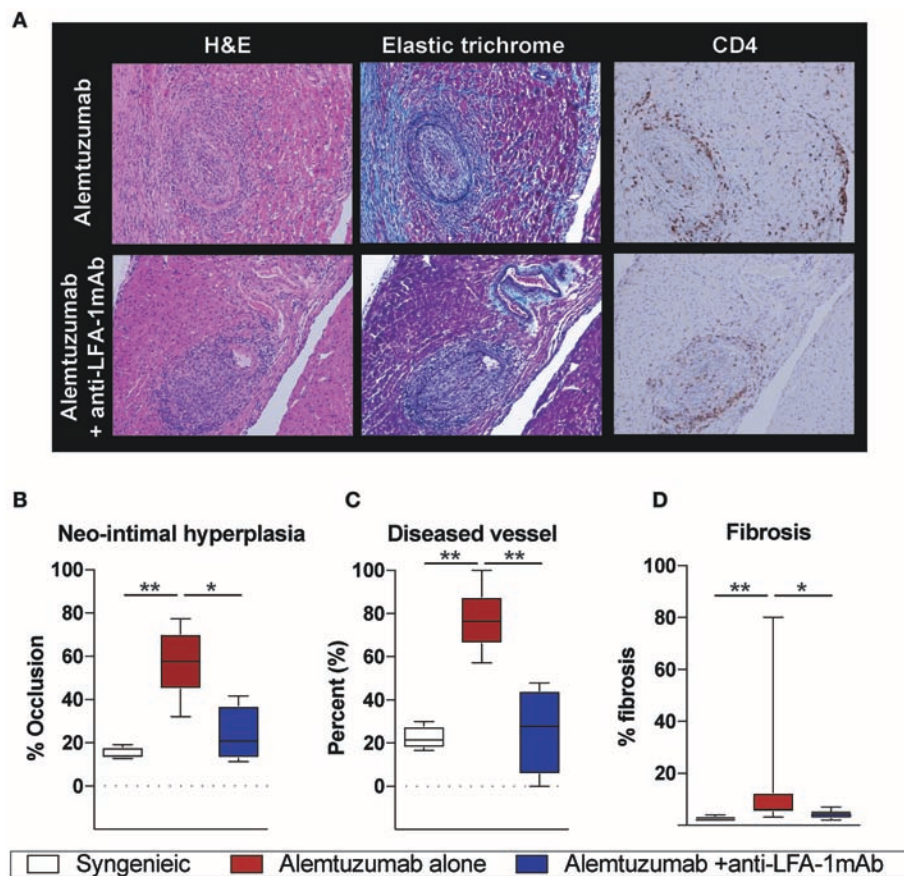


FIGURE 2 | Alemtuzumab induced CAMR in human CD52 Tg cardiac allograft recipient was diminished by short-term anti-LFA-1 mAb treatment. **(A)** Representative images of H and E, Elastic trichrome, and CD4 staining in paraffin section of heart allograft from recipient showing neo-intimal hyperplasia. **(B–D)** Morphometric quantitation of lumina occlusion, occluded vessel number (over 20%), fibrosis of explanted graft from B6 heart allografts revealed reduction of CAMR with additional LFA-1 blockade. * $p < 0.05$, ** $p < 0.01$.

interaction in the B cell follicle may be altered by anti-LFA-1 mAb. This may result in reduced development of follicular helper T cells and consequent lack of allo-B cell clonal expansion.

Post-transplant Germinal Center Suppression via Blocking LFA-1

Based on a reduction of allo-B cells and IL-21/IL-4 production following anti-LFA-1 mAb treatment, we hypothesized that LFA-1 blockade suppresses GC-Tfh cell development, B cell clonal expansion, and GC development. We evaluated GC Tfh cell responses *in situ* from lymph nodes on POD 100. Lymph nodes from naïve, alemtuzumab-alone—treated, and additional anti-LFA-1 mAb-treated recipients were stained with H and E, CD3, B220, Ki67, PNA, and IL-21. T and B cell zones were equally reconstituted in both treated groups at POD 100. Multiple hyperplastic GCs were found in samples from mice treated with alemtuzumab-alone ($n = 7$), but LFA-1 blockade reduced GC frequency and size to baseline (naïve) levels ($n = 9$). We also found that IL-21 staining was greatly reduced in the anti-LFA-1 mAb-treated group (Figure 5).

DISCUSSION

In transplantation, the relationship of DSA with AMR and graft outcome is a subject of much clinical study. In the clinical setting, DSA are generally detrimental to transplant outcomes via complement activation, induction of endothelial cell proliferation, and ADCC resulting in allograft dysfunction (AMR). Despite this, little is known about the mechanism by which the humoral response is induced under current clinical immunosuppression. We published a series of studies showing a possible involvement of Tfh and GC response in the secondary lymphoid organ in transplantation and antibody-mediated rejection in small and large animal models (15, 21). However, the biology of Tfh cell mediated B cell activation has not been fully elucidated in transplantation. In autoimmune diseases, unwanted Tfh dysregulation is associated with autoimmunity, and increased frequencies of peripheral Tfh cells have been reported in several autoimmune diseases, such as lupus, Sjögren syndrome, autoimmune thyroiditis, myasthenia gravis and rheumatoid arthritis (35–39). On the other hand, the expansion of Tfh cells are associated with efficacious vaccine strategies

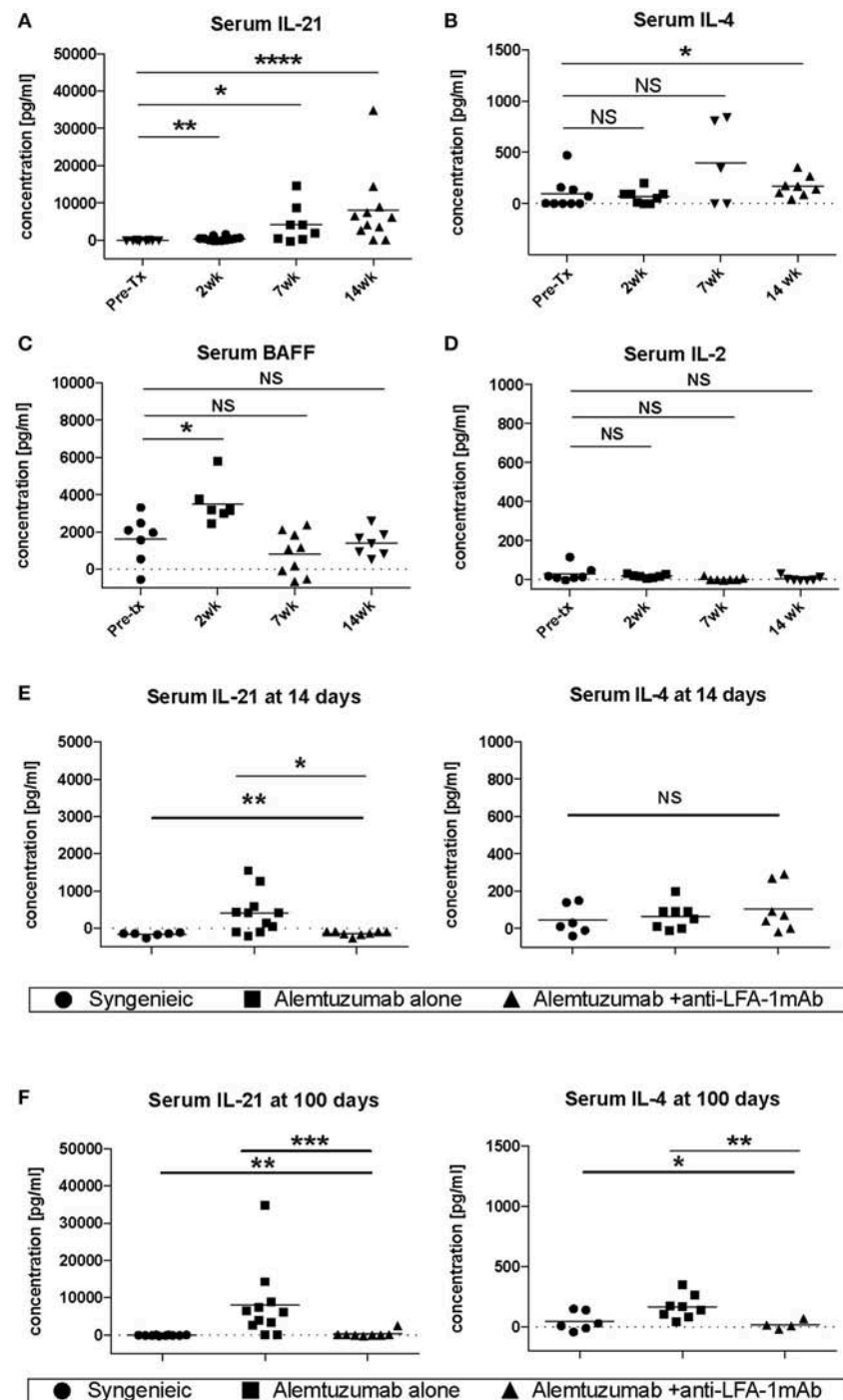


FIGURE 3 | Systemic cytokine expression in human CD52Tg cardiac allograft recipients after alemtuzumab treatment. Pre- and Post-transplant level of (A) IL-21, (B) IL-4, (C) BAFF, and (D) IL-2 were measured from serum. IL-4 and IL-21 levels were significantly reduced by anti-LFA-1 mAb treatment at (E) Post-transplant 2 weeks and (F) post-transplant 14 weeks. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

(40, 41). Autoimmune diseases are also often characterized by decreased subset of Tregs called follicular regulatory T cells (Tfr cells). Tfr cells express CXCR5 and FOXP3, and suppress B cell antibody production (42).

The hallmark cytokine produced by Tfh cells is IL-21. IL-21 is a gamma-chain cytokine with broad effects on both innate and adaptive immune responses (43). Significantly, IL-21 is required for the generation of Tfh cells (44, 45).

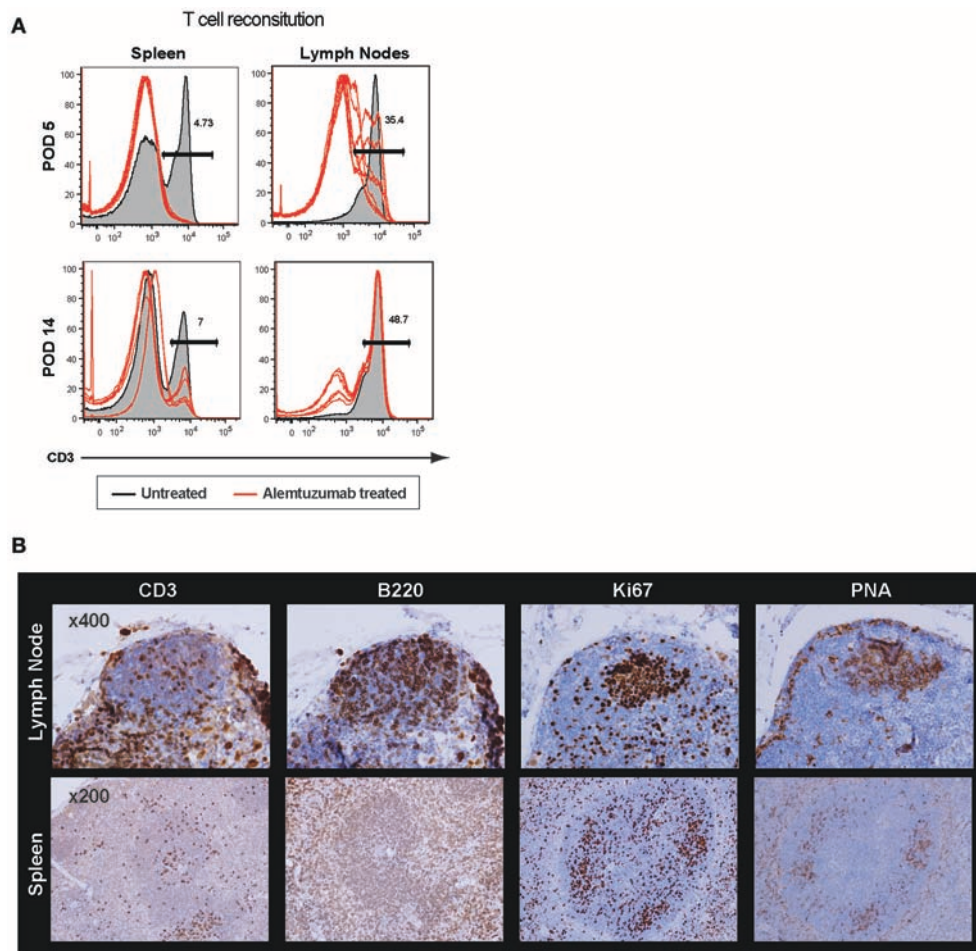


FIGURE 4 | Incomplete germinal center disruption after alemtuzumab treatment in the lymph node and spleen. **(A)** Significantly higher frequency of CD3 T cells were found in the lymph nodes compared to other immune compartments at POD 5 and POD 14 (Gray area = non-depleted control, Red line = alemtuzumab treated). **(B)** Immunohistochemistry demonstrated cell components of germinal center. T, B, Ki67 and PNA staining confirmed germinal center (GC) response at 24 h after alemtuzumab treatment.

Interestingly, IL-21 can overcome Tfr suppression to induce B cell activation, thereby demonstrating a critical role for IL-21 in dictating the germinal center response. In transplantation, it has been shown that transplant recipients with preformed DSA showed higher post-transplant circulating Tfh cell (46). Furthermore, alloantigen stimulated Tfh cell population promoted B cell differentiation to plasmablast in an IL-21 dependent manner *in vitro* (47). It is also shown the intact GC response from isolated lymphoid follicles under an immunosuppressive regimen with tacrolimus in intestinal transplant recipient (48). Tfh and GC response were also required for chronic GvHD and inhibition of GC response greatly reduced chronic GvHD (49, 50). Collectively, the presence of IL-21 in secondary lymphoid organs or in local tertiary lymphoid structures can be detrimental to transplant tolerance due to its impact on the generation of Tfh cells, its autocrine production, and on B cell maturation and antibody production.

Unfortunately, there are no animal models demonstrating a correlation between Tfh/GC/IL-21 to the development of chronic rejection, even though, CAMR is more dependent on GC response, with critical help provided by Tfh cells. We have reported the *de novo* AMR model using alemtuzumab mediated T cell depletion with heterotopic heart transplantation (20). As follow-up studies, we tried additional immunosuppression in the model to suppress post-transplant humoral response. Counterintuitively, the addition of short-term tacrolimus (data not shown) or rapamycin (21) did not alleviate post-transplant humoral response but rather made it worse (increased DSA and AMR). As previously reported, costimulation blockade in the mouse CAMR model reversed this by reducing Tfh cells (21). In the present study, we targeted LFA-1, which is well known for their important role for T-B conjugation. We investigated the possible mechanism of CAMR by using anti-LFA-1 mAb in an Ab-dependent rejection model, independent of T cell mediated rejection.

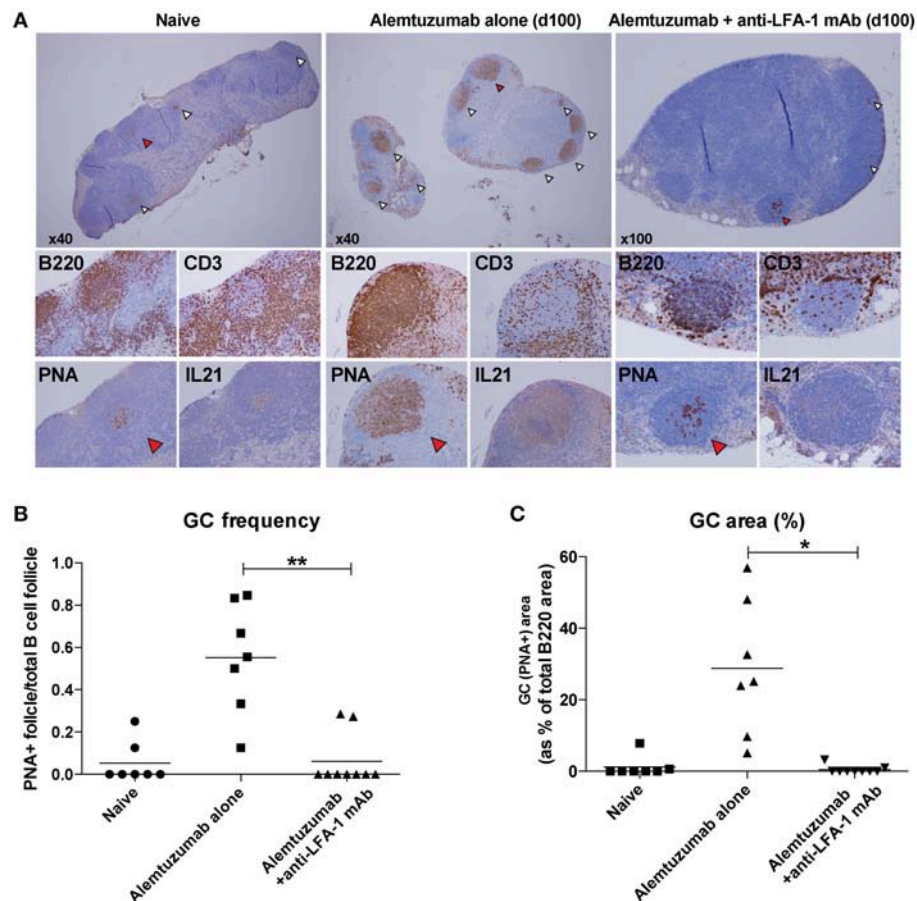


FIGURE 5 | Reduction of post-transplant GC response with anti-LFA-1 mAb treatment. **(A)** post-transplant GC response was visualized by B220, CD3, PNA, and IL-21 staining *in situ*. **(B)** GC frequency was quantified by calculating the PNA⁺ follicle number by total B220⁺ follicle number. **(C)** GC response or hyperplasticity was measured by calculating PNA⁺ area by total B220 area. * $p < 0.05$, ** $p < 0.01$.

As shown in **Figure 1**, additional anti-LFA-1 mAb treatment completely abolished post-transplant DSA and donor-specific B cells were strongly correlative with alleviation of AMR development. Similar to acute NHP AMR model using T cell depletion (15), CD52Tg mice with alemtuzumab showed profound circulating T cell depletion while T cells remained in the lymph nodes and spleen. We thoroughly access the location of T cells in the lymph node and spleen after T cell depletion (24h and 7 days). It is quite surprising that the germinal centers including T cells in the B cell follicle are preferentially identified after T cell depletion (**Figure 4**). In accordance with this, Kirk et al. reported presence of T cells in the lymph node after alemtuzumab treatment (31). Interestingly, we also identified hyperplastic germinal center at POD100 with elevated level of DSA and AMR (**Figure 5**). As previously reported, T cells are fully reconstructed after alemtuzumab yet show donor-specific hyporesponsiveness (20). Based on this, the elevated level of DSA can directly cause graft injury (**Figure 1**). On the contrary, anti-LFA-1 mAb treated recipients showed baseline level of serum DSA, allo-specific B

cells and germinal center response at POD 100 (**Figures 1, 5**). It is conjectured that these left over Tfh cell in the germinal center could deviate the systematic cytokine milieu that alemtuzumab treated recipients showed significantly elevated level of IL-21 in their serum (**Figure 3**). Taken together, the present study demonstrates that GC-dependent CAMR development after alemtuzumab treatment and anti-LFA-1 mAb treatment can prevent the development of post-transplant humoral response and CAMR.

Here, we assumed that anti-LFA-1 mAb is dissociating T-B cell conjugation during T cell repopulation, however, the impact of LFA-1 blockade cannot be limited to this since LFA-1 is not solely expressed on T and B cells (51). It is known that anti-LFA-1 mAb prevents allo-specific T cell expansion (52) and promote transplant tolerance (53–55) by destabilizing T-APC conjugation as well as blocking transmigration to the graft. Therefore, anti-LFA-1 mAb can reduce Tfh cell population by suppressing general T cell activation and expansion. Nevertheless (Regardless of off target effect of anti-LFA-1 mAb), this mode of action can block the T cell help to the B cells.

Unfortunately, clinical use of anti-LFA-1 mAb is not available in clinic for organ transplantation. However, the present study showing reduction of DSA and CAMR with anti-LFA-1 mAb recapitulate the impact of costimulation blockade in large animal model and clinic (15, 16, 56). Costimulation blockade such as belatacept often show great superiority in suppressing DSA production. It is highly likely that Tfh cells are more sensitive on belatacept than other conventional immunosuppressive drugs. However, it is still not clear that anti-LFA-1 mAb and other costimulation blockades are working on pre-GC vs. post-GC status. Overall, Tfh cells are very attractive target for controlling post-transplant humoral response.

In this study, we identify a possible mechanistic pathway that regulates a *de novo* DSA response after cytolytic induction and provide a strategy for modulating the post-transplant humoral response. In particular, the ability to suppress the functional qualities of follicular helper T cells by costimulation blockade provides a new approach to induce humoral unresponsiveness in organ transplantation.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the guidelines set forth by the National Institutes of Health and Office of Laboratory Animal Welfare. All medications and procedures were reviewed and approved by the Emory or Duke Institutional Animal Research Ethics Committee (IACUC).

REFERENCES

- Rahmani M, Cruz RP, Granville DJ, McManus BM. Allograft vasculopathy versus atherosclerosis. *Circ Res.* (2006) 99:801–15. doi: 10.1161/01.RES.0000246086.93555.f3
- Costanzo MR, Naftel DC, Pritzker MR, Heilman JK III, Boehmer JP, Brozena SC, et al. Heart transplant coronary artery disease detected by coronary angiography: a multiinstitutional study of preoperative donor and recipient risk factors. Cardiac Transplant Research Database. *J Heart Lung Transplant.* (1998) 17:744–53.
- Kobashigawa J, Wener L, Johnson J, Currier JW, Yeatman L, Cassem J, et al. Longitudinal study of vascular remodeling in coronary arteries after heart transplantation. *J Heart Lung Transplant.* (2000) 19:546–50. doi: 10.1016/S1053-2498(00)00100-5
- McKenna RM, Takemoto SK, Terasaki PI. Anti-HLA antibodies after solid organ transplantation. *Transplantation* (2000) 69:319–26. doi: 10.1097/00007890-200002150-00001
- Girnit AL, McCurry KR, Zeevi A. Increased lung allograft failure in patients with HLA-specific antibody. *Clin Transpl.* (2007) 231–9.
- Tan CD, Baldwin WM III, Rodriguez ER. Update on cardiac transplantation pathology. *Arch Pathol Lab Med.* (2007) 131:1169–91. doi: 10.1043/1543-2165(2007)131[1169:UOCTP]2.0.CO;2
- Terasaki P, Lachmann N, Cai J. Summary of the effect of *de novo* HLA antibodies on chronic kidney graft failure. *Clin Transpl.* (2006) 455–62.
- Terasaki PI, Cai J. Human leukocyte antigen antibodies and chronic rejection: from association to causation. *Transplantation* (2008) 86:377–83. doi: 10.1097/TP.0b013e31817c4cb8
- Calne R, Friend P, Moffatt S, Bradley A, Hale G, Firth J, et al. Prope tolerance, perioperative campath 1H, and low-dose cyclosporin

AUTHOR CONTRIBUTIONS

JK conceived the idea, designed experiments, and analyzed data. JK and JP performed mouse experiments. JK and JY performed *in vitro* experiments. AF read pathology. AK provided critical reagents and wrote the paper. JK and SK wrote the paper.

FUNDING

This work was supported by American Heart Association (AHA)/Enduring Heart Foundation Research Award 15SDG25710165 awarded to JK.

ACKNOWLEDGMENTS

We thank Drew Roenneburg (University of Wisconsin-Madison) for performing immunohistochemistry and Miriam Manook (Department of Surgery, King's college, UK) for critical manuscript review. We also thank Stephanie Freel and Ashley Morgan for their critiques on the manuscript. We also thank Sanofi S. A. for generously provided alemtuzumab for the study. MHC Class I monomers were provided by the NIH Tetramer Core Facility.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2018.02323/full#supplementary-material>

- monotherapy in renal allograft recipients. *Lancet* (1998) 351:1701–2. doi: 10.1016/S0140-6736(05)77739-4
- Swanson SJ, Hale DA, Mannon RB, Kleiner DE, Cendales LC, Chamberlain CE, et al. Kidney transplantation with rabbit antithymocyte globulin induction and sirolimus monotherapy. *Lancet* (2002) 360:1662–4. doi: 10.1016/S0140-6736(02)11606-0
- Hanaway MJ, Woodle ES, Mulgaonkar S, Peddi VR, Kaufman DB, First MR, et al. Alemtuzumab induction in renal transplantation. *N Engl J Med.* (2011) 364:1909–19. doi: 10.1056/NEJMoa1009546
- Bloom DD, Chang Z, Fechner JH, Dar W, Polster SP, Pascual J, et al. CD4⁺ CD25⁺ FOXP3⁺ regulatory T cells increase *de novo* in kidney transplant patients after immunodepletion with Campath-1H. *Am J Transplant.* (2008) 8:793–802. doi: 10.1111/j.1600-6143.2007.02134.x
- Knechtle SJ, Pascual J, Bloom DD, Torrealba JR, Jankowska-Gan E, Burlingham WJ, et al. Early and limited use of tacrolimus to avoid rejection in an alemtuzumab and sirolimus regimen for kidney transplantation: clinical results and immune monitoring. *Am J Transplant.* (2009) 9:1087–98. doi: 10.1111/j.1600-6143.2009.02581.x
- Willicombe M, Brookes P, Santos-Nunez E, Galliford J, Ballow A, McLean A, et al. Outcome of patients with preformed donor-specific antibodies following alemtuzumab induction and tacrolimus monotherapy. *Am J Transplant.* (2011) 11:470–7. doi: 10.1111/j.1600-6143.2010.03421.x
- Kim EJ, Kwun J, Gibby AC, Hong JJ, Farris AB III, Iwakoshi NN, et al. Costimulation blockade alters germinal center responses and prevents antibody-mediated rejection. *Am J Transplant.* (2014) 14:59–69. doi: 10.1111/ajt.12526
- Leibler C, Thiolat A, Henique C, Samson C, Pilon C, Tamagne M, et al. Control of humoral response in renal transplantation by belatacept depends on a direct effect on B cells and impaired T follicular helper-B cell crosstalk. *J Am Soc Nephrol.* (2018) 29:1049–62. doi: 10.1681/ASN.201706067

17. Kwun J, Malarkannan S, Burlingham WJ, Knechtle SJ. Primary vascularization of the graft determines the immunodominance of murine minor H antigens during organ transplantation. *J Immunol.* (2011) 187:3997–4006. doi: 10.4049/jimmunol.1003918
18. Billingham ME, Cary NR, Hammond ME, Kemnitz J, Marboe C, McCallister HA, et al. A working formulation for the standardization of nomenclature in the diagnosis of heart and lung rejection: Heart Rejection Study Group. The International Society for Heart Transplantation. *J Heart Transplant.* (1990) 9:587–93.
19. Stewart S, Winters GL, Fishbein MC, Tazelaar HD, Kobashigawa J, Abrams J, et al. Revision of the 1990 working formulation for the standardization of nomenclature in the diagnosis of heart rejection. *J Heart Lung Transplant.* (2005) 24:1710–20. doi: 10.1016/j.healun.2005.03.019
20. Kwun J, Oh BC, Gibby AC, Ruhil R, Lu VT, Kim DW, et al. Patterns of *de novo* allo B cells and antibody formation in chronic cardiac allograft rejection after alemtuzumab treatment. *Am J Transplant.* (2012) 12:2641–51. doi: 10.1111/j.1600-6143.2012.04181.x
21. Oh B, Yoon J, Farris A III, Kirk A, Knechtle S, Kwun J. Rapamycin interferes with postdepletion regulatory T cell homeostasis and enhances DSA formation corrected by CTLA4-Ig. *Am J Transplant.* (2016) 16:2612–23. doi: 10.1111/ajt.13789
22. Soleimani B, Wiecek G, Katopodis A, Zenke G, George AJ, Hornick PI, et al. Anti-LFA-1 monotherapy prevents neointimal formation in a murine model of transplant intimal hyperplasia. *J Heart Lung Transplant.* (2007) 26:724–31. doi: 10.1016/j.healun.2007.04.007
23. Shimizu K, Shichiri M, Libby P, Lee RT, Mitchell RN. Th2-predominant inflammation and blockade of IFN-gamma signaling induce aneurysms in allografted aortas. *J Clin Invest.* (2004) 114:300–8. doi: 10.1172/JCI200419855
24. Paul WE, Ohara J. B-cell stimulatory factor-1/interleukin 4. *Annu Rev Immunol.* (1987) 5:429–59. doi: 10.1146/annurev.iy.05.040187.002241
25. Fazilleau N, Mark L, McHeyzer-Williams LJ, McHeyzer-Williams MG. Follicular helper T cells: lineage and location. *Immunity* (2009) 30:324–35. doi: 10.1016/j.immuni.2009.03.003
26. Ozaki K, Spolski R, Feng CG, Qi CF, Cheng J, Sher A, et al. A critical role for IL-21 in regulating immunoglobulin production. *Science* (2002) 298:1630–4. doi: 10.1126/science.1077002
27. Bloom D, Chang Z, Pauly K, Kwun J, Fechner J, Hayes C, et al. BAFF is increased in renal transplant patients following treatment with alemtuzumab. *Am J Transplant.* (2009) 9:1835–45. doi: 10.1111/j.1600-6143.2009.02710.x
28. Todeschini M, Cortinovis M, Perico N, Poli F, Innocente A, Cavinato RA, et al. In kidney transplant patients, alemtuzumab but not basiliximab/low-dose rabbit anti-thymocyte globulin induces B cell depletion and regeneration, which associates with a high incidence of *de novo* donor-specific anti-HLA antibody development. *J Immunol.* (2013) 191:2818–28. doi: 10.4049/jimmunol.1203261
29. Ballesteros-Tato A, Leon B, Graf BA, Moquin A, Adams PS, Lund FE, et al. Interleukin-2 inhibits germinal center formation by limiting T follicular helper cell differentiation. *Immunity* (2012) 36:847–56. doi: 10.1016/j.immuni.2012.02.012
30. Yusuf I, Kageyama R, Monticelli L, Johnston RJ, Ditoro D, Hansen K, et al. Germinal center T follicular helper cell IL-4 production is dependent on signaling lymphocytic activation molecule receptor (CD150). *J Immunol* (2010) 185:190–202. doi: 10.4049/jimmunol.0903505
31. Kirk AD, Hale DA, Mannon RB, Kleiner DE, Hoffmann SC, Kampen RL, et al. Results from a human renal allograft tolerance trial evaluating the humanized CD52-specific monoclonal antibody alemtuzumab (CAMPATH-1H). *Transplantation* (2003) 76:120–9. doi: 10.1097/01.TP.0000071362.99021.D9
32. Korn T, Bettelli E, Gao W, Awasthi A, Jäger A, Strom TB, et al. IL-21 initiates an alternative pathway to induce proinflammatory T(H)17 cells. *Nature* (2007) 448:484–7. doi: 10.1038/nature05970
33. Kwun J, Manook M, Page E, Burghuber C, Hong J, Knechtle SJ. Crosstalk between T and B cells in the germinal center after transplantation. *Transplantation* (2017) 101:704–12. doi: 10.1097/TP.0000000000001588
34. Kurzinger K, Reynolds T, Germain RN, Davignon D, Martz E, Springer TA. A novel lymphocyte function-associated antigen (LFA-1): cellular distribution, quantitative expression, and structure. *J Immunol.* (1981) 127:596–602.
35. Hutloff A, Buchner K, Reiter K, Baelde HJ, Odendahl M, Jacobi A, et al. Involvement of inducible costimulator in the exaggerated memory B cell and plasma cell generation in systemic lupus erythematosus. *Arthritis Rheum.* (2004) 50:3211–20. doi: 10.1002/art.20519
36. Li XY, Wu ZB, Ding J, Zheng ZH, Li XY, Chen LN, et al. Role of the frequency of blood CD4⁺ CXCR5⁺ CCR6⁺ T cells in autoimmunity in patients with Sjogren's syndrome. *Biochem Biophys Res Commun.* (2012) 422:238–44. doi: 10.1016/j.bbrc.2012.04.133
37. Ma J, Zhu C, Ma B, Tian J, Baidoo SE, Mao C, et al. Increased frequency of circulating follicular helper T cells in patients with rheumatoid arthritis. *Clin Dev Immunol.* (2012) 2012:827480. doi: 10.1155/2012/827480
38. Zhu C, Ma J, Liu Y, Tong J, Tian J, Chen J, et al. Increased frequency of follicular helper T cells in patients with autoimmune thyroid disease. *J Clin Endocrinol Metab.* (2012) 97:943–50. doi: 10.1210/jc.2011-2003
39. Saito R, Onodera H, Tago H, Suzuki Y, Shimizu M, Matsumura Y, et al. Altered expression of chemokine receptor CXCR5 on T cells of myasthenia gravis patients. *J Neuroimmunol.* (2005) 170:172–8. doi: 10.1016/j.jneuroim.2005.09.001
40. Matsui K, Adelsberger JW, Kemp TJ, Baseler MW, Ledgerwood JE, Pinto LA. Circulating CXCR5⁺ CD4⁺ T follicular-like helper cell and memory B cell responses to human papillomavirus vaccines. *PLoS ONE* (2015) 10:e0137195. doi: 10.1371/journal.pone.0137195
41. Pilkinton MA, Nicholas KJ, Warren CM, Smith RM, Yoder SM, Talbot HK, et al. Greater activation of peripheral T follicular helper cells following high dose influenza vaccine in older adults forecasts seroconversion. *Vaccine* (2017) 35:329–36. doi: 10.1016/j.vaccine.2016.11.059
42. Sage PT, Ron-Harel N, Juneja VR, Sen DR, Maleri S, Sungnak W, et al. Suppression by TFR cells leads to durable and selective inhibition of B cell effector function. *Nat Immunol* (2016) 17:1436–46. doi: 10.1038/ni.3578
43. Yi JS, Cox MA, Zajac AJ. Interleukin-21: a multifunctional regulator of immunity to infections. *Microbes Infect* (2010) 12:1111–9. doi: 10.1016/j.micinf.2010.08.008
44. Nurieva RI, Chung Y, Hwang D, Yang XO, Kang HS, Ma L, et al. Generation of T follicular helper cells is mediated by interleukin-21 but independent of T helper 1, 2, or 17 cell lineages. *Immunity* (2008) 29:138–49. doi: 10.1016/j.immuni.2008.05.009
45. Vogelzang A, McGuire HM, Yu D, Sprent J, Mackay CR, King C. A fundamental role for interleukin-21 in the generation of T follicular helper cells. *Immunity* (2008) 29:127–37. doi: 10.1016/j.immuni.2008.06.001
46. de Graaf GN, Dieterich M, Hesselink DA, Boer K, Clahsen-van Groningen MC, Kraaijeveld R, et al. Follicular T helper cells and humoral reactivity in kidney transplant patients. *Clin Exp Immunol.* (2015) 180:329–40. doi: 10.1111/cei.12576
47. de Leur K, Dor FJ, Dieterich M, van der Laan LJ, Hendriks RW, Baan CC. IL-21 Receptor antagonist inhibits differentiation of B cells toward plasmablasts upon alloantigen stimulation. *Front Immunol.* (2017) 8:306. doi: 10.3389/fimmu.2017.00306
48. Meier D, Docena GH, Ramisch D, Toscanini U, Berardi G, Gondolesi GE, et al. Immunological status of isolated lymphoid follicles after intestinal transplantation. *Am J Transplant.* (2014) 14:2148–58. doi: 10.1111/ajt.12797
49. Srinivasan M, Flynn R, Price A, Ranger A, Browning JL, Taylor PA, et al. Donor B-cell alloantibody deposition and germinal center formation are required for the development of murine chronic GVHD and bronchiolitis obliterans. *Blood* (2012) 119:1570–80. doi: 10.1182/blood-2011-07-364414
50. Flynn R, Du J, Veenstra RG, Reichenbach DK, Panoskaltis-Mortari A, Taylor PA, et al. Increased T follicular helper cells and germinal center B cells are required for cGVHD and bronchiolitis obliterans. *Blood* (2014) 123:3988–98. doi: 10.1182/blood-2014-03-562231
51. Fawcett J, Holness CL, Needham LA, Turley H, Gatter KC, Mason DY, et al. Molecular cloning of ICAM-3, a third ligand for LFA-1, constitutively expressed on resting leukocytes. *Nature* (1992) 360:481–4. doi: 10.1038/360481a0
52. Kwun J, Farris AB, Song H, Mahle WT, Burlingham WJ, Knechtle SJ. Impact of leukocyte function-associated antigen-1 blockade on endogenous allospecific T cells to multiple minor histocompatibility antigen mismatched cardiac allograft. *Transplantation* (2015) 99:2485–93. doi: 10.1097/TP.0000000000000805

53. Springer TA, Dustin ML, Kishimoto TK, Marlin SD. The lymphocyte function-associated LFA-1, CD2, and LFA-3 molecules: cell adhesion receptors of the immune system. *Annu Rev Immunol.* (1987) 5:223–52. doi: 10.1146/annurev.iy.05.040187.001255
54. Lee KH, Holdorf AD, Dustin ML, Chan AC, Allen PM, Shaw AS. T cell receptor signaling precedes immunological synapse formation. *Science* (2002) 295:1539–42. doi: 10.1126/science.1067710
55. Isobe M, Yagita H, Okumura K, Ihara A. Specific acceptance of cardiac allograft after treatment with antibodies to ICAM-1 and LFA-1. *Science* (1992) 255:1125–7. doi: 10.1126/science.1347662
56. Xu H, Samy KP, Guasch A, Mead SI, Ghali A, Mehta A, et al. Postdepletion lymphocyte reconstitution during belatacept and rapamycin

treatment in kidney transplant recipients. *Am J Transplant.* (2016) 16:550–64. doi: 10.1111/ajt.13469

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2018 Kwun, Park, Yi, Farris, Kirk and Knechtle. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



Molecular Control of Follicular Helper T cell Development and Differentiation

Haijing Wu¹, Yaxiong Deng^{1,2}, Ming Zhao¹, Jianzhong Zhang³, Min Zheng⁴, Genghui Chen⁵, Linfeng Li⁶, Zhibiao He⁷ and Qianjin Lu^{1*}

¹ Hunan Key Laboratory of Medical Epigenomics, Department of Dermatology, Second Xiangya Hospital, Central South University, Changsha, China, ² Immunology Section, Lund University, Lund, Sweden, ³ Department of Dermatology, Peking University People's Hospital, Beijing, China, ⁴ Department of Dermatology, The Second Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou, China, ⁵ Beijing Wenfeng Tianji Pharmaceuticals Ltd., Beijing, China, ⁶ Department of Dermatology, Beijing Friendship Hospital, Capital Medical University, Beijing, China, ⁷ Department of Emergency, Second Xiangya Hospital of Central South University, Changsha, China

OPEN ACCESS

Edited by:

Shahram Salek-Ardakani,
Pfizer, United States

Reviewed by:

Betty Diamond,
Feinstein Institute for Medical
Research, United States
Richard Lee Reinhardt,
National Jewish Health, United States
Sun Jung Kim,
Northwell Health, United States, in
collaboration with reviewer BD

*Correspondence:

Qianjin Lu
qianlu5860@gmail.com;
qianlu5860@csu.edu.cn

Specialty section:

This article was submitted to
T cell Biology,
a section of the journal
Frontiers in Immunology

Received: 15 June 2018

Accepted: 05 October 2018

Published: 25 October 2018

Citation:

Wu H, Deng Y, Zhao M, Zhang J,
Zheng M, Chen G, Li L, He Z and
Lu Q (2018) Molecular Control of
Follicular Helper T cell Development
and Differentiation.
Front. Immunol. 9:2470.
doi: 10.3389/fimmu.2018.02470

Follicular helper T cells (T_{fh}) are specialized helper T cells that are predominantly located in germinal centers and provide help to B cells. The development and differentiation of T_{fh} cells has been shown to be regulated by transcription factors, such as B-cell lymphoma 6 protein (Bcl-6), signal transducer and activator of transcription 3 (STAT3) and B lymphocyte-induced maturation protein-1 (Blimp-1). In addition, cytokines, including IL-21, have been found to be important for T_{fh} cell development. Moreover, several epigenetic modifications have also been reported to be involved in the determination of T_{fh} cell fate. The regulatory network is complicated, and the number of novel molecules demonstrated to control the fate of T_{fh} cells is increasing. Therefore, this review aims to summarize the current knowledge regarding the molecular regulation of T_{fh} cell development and differentiation at the protein level and at the epigenetic level to elucidate T_{fh} cell biology and provide potential targets for clinical interventions in the future.

Keywords: T_{fh}, Bcl-6, Blimp-1, transcription factors, epigenetics

INTRODUCTION

A subset of CD4⁺ T cells, which help B cells and are a resident in B follicles, has been described in the early 1990s (1–4). The existence of follicular helper T (T_{fh}) cells was proposed in 2000 (5, 6). However, the existence of these cells was not widely accepted until the identification of the T_{fh} cell lineage-specific transcription factor, B-cell lymphoma 6 protein (Bcl-6), in follicular T cells in 2009 (7, 8). High expression of CXCR5 and low expression of CCR7 enable T cells to enter and stay in germinal centers (GCs) (6, 9–11). Bcl-6 deficient T cells have been shown to fail to differentiate into follicular helper T cells (8), indicating the importance of Bcl-6 in the determination of T_{fh} cell fate. Under the effects of CCL19 and CCL21, expression of the receptor CCR7 on naïve CD4⁺ T cells enables these cells to migrate into T cell zones in the secondary lymph nodes (9, 12). With stimulation from antigens and CD80, CD86 and ICOSL expressed on dendritic cells (DCs), these cells differentiate into pre-T_{fh} cells with high expression of PD-1, CXCR5 and signaling lymphocytic activation molecule adapter protein (SAP) (13) and low expression of CCR7 and P selectin glycoprotein ligand 1 (PSGL1) (14, 15) (**Figure 1**). Generally, T_{fh} cells provide signals for B cell maturation, differentiation and survival via ICOS, CD40L, IL-4, and IL-21 (16, 17). ICOS and

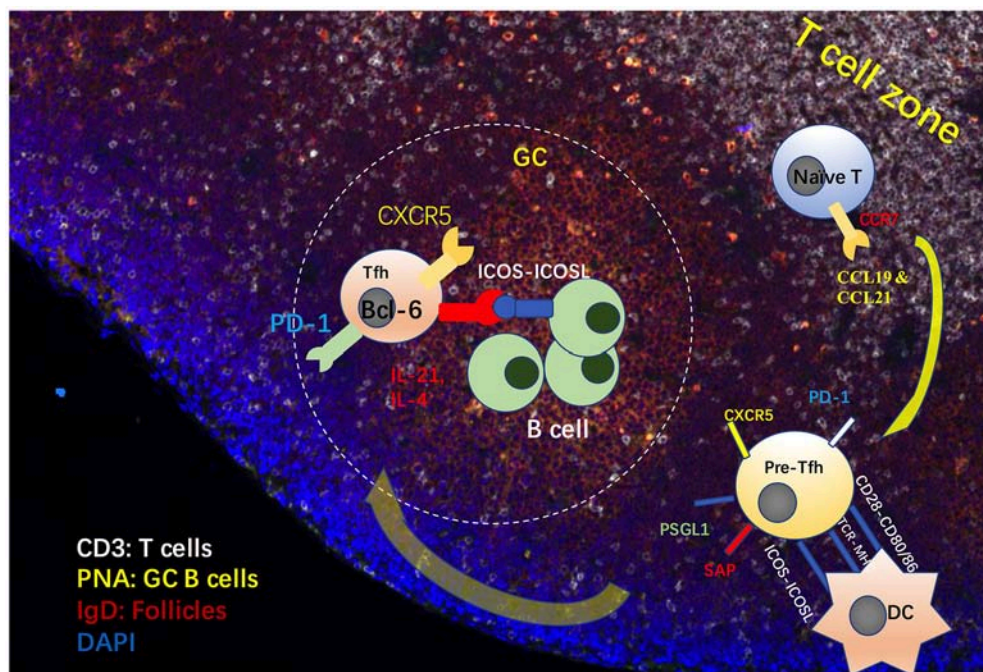


FIGURE 1 | Process of Tfh cell differentiation and migration in GCs. Under the effects of CCL19 and CCL21, expression of the receptor CCR7 on naïve CD4⁺ T cells allows these cells to migrate into T cell zones in the secondary lymph nodes. With stimulation from antigens and CD80, CD86 and ICOSL expressed on dendritic cells (DCs), these cells differentiate into pre-Tfh cells, with high expression of CXCR5, PD-1 and signaling lymphocytic activation molecule adapter protein (SAP) and low expression of CCR7 and P selectin glycoprotein ligand 1 (PSGL1). Generally, Tfh cells provide signals for B cell maturation, differentiation and survival via ICOS, CD40L, IL-4, and IL-21 (16, 17). ICOS and ICOSL ligation is involved in Tfh-B cell interactions, which promotes calcium spikes in T-cells and CD40-CD40L signaling in B cells.

ICOSL ligation is involved in T-B cell interactions, further promoting calcium spikes in T-cells and CD40-CD40L signaling in B cells (18). ICOS-deficient T cells fail to express CXCR5 and are unable to migrate into follicles, a finding also observed during antibody-blockade of ICOS-ligand (19, 20). PD-1 has been found to limit the number of Tfh cells (21). More evidence is needed to address the role of PD-1 in the migration and function of Tfh cells. SAP has been found to stabilize the interaction between B cells and Tfh cells (22). Therefore, Tfh cells can be distinguished from Th1, Th2 and Th17 cells using surface markers with a profile of CCR7^{lo}PSGL1^{lo}CXCR5^{hi}PD-1^{hi}ICOS^{hi}. Activated by antigens and ICOSL expressed by DCs, the expression of Bcl-6 is upregulated in CD4⁺ T cells, and it represses other Th cell transcription factors, such as T-bet, GATA-3, and RORγT. Next, Bcl-6 promotes the transcription of Tfh cell migration and function-related genes, such as CXCR5, PD-1, and CXCR4 (8).

Tfh cells have been found to be regulated by a complex network of transcription factors, including the Bcl-6-Blimp1 axis, STAT1, STAT3, STAT4, STAT5b, B-cell activating transcription factor (Batf), v-maf avian musculoaponeurotic fibrosarcoma oncogene homolog (c-Maf), interferon regulatory factor 4 (IRF4), Achaete-scute homolog 2 (Acl2), and T-cell-specific transcription factor 1 (TCF-1)-lymphoid enhancer binding factor 1 (LEF-1). Since the study of Tfh cells began, certain proteins have been identified to participate in the development of Tfh cells. In addition, such cytokines as IL-2, IL-6, IL-7, IL-9, IL-12,

IL-21, IL-23, IL-27, and TGF-β have been reported to enhance or impair the differentiation and survival of Tfh cells. Therefore, this review will comprehensively describe the current knowledge of Tfh cells, hoping to provide potential targets for Tfh cell-mediated autoimmune disease.

Transcription Factor Network

Bcl-6-Blimp-1 Axis

The discovery of Bcl-6 in Tfh cells is a hallmark for the identification of Tfh cells. The essential role of Bcl-6 has been confirmed in a mice study, indicating that CD4⁺ T cells deficient in Bcl-6 fail to differentiate into Tfh cells (8). Forced expression of Bcl-6 in CD4⁺ T cells promotes the expression of CXCR5, CXCR4, and PD-1 (8). Bcl-6 can bind to the promoters of Th1 and Th17 cell transcriptional regulators T-bet and RORγT, thereby repressing the production of IFN-γ and IL-17 (8). The key role of Bcl-6 in Tfh cell fate determination has been further confirmed in subsequent studies (23, 24), and one of them reveals that Bcl-6 regulates Tfh cell early differentiation in an IL-21- and IL-6-independent manner (24). Conversely, Bcl-6 can bind to the promoters and enhancers of several migration-related genes, such as CCR7, CCR6, PSGL-1, CXCR5, CXCR4, PD-1, and SAP (24, 25). In addition, Bcl-6-targeted genes are enriched in the MAPK and JAK-STAT signaling pathways and cytokine-cytokine receptor ligations, which are involved in cell activation, metabolism and maintenance (26).

Blimp-1 has been found to be a critical antagonist for Tfh cell differentiation but an important transcription factor for other effector cells, such as Th1, Th2, Th17, and regulatory T cells (7). In a mouse study, Blimp-1-deficient CD4⁺ T cells preferentially develop into Tfh cells *in vivo*, while Blimp-1-expressing CD4⁺ T cells failed to aid in germinal center formation (7). Therefore, Tfh cell differentiation is believed to be a distinct pathway independently regulated by Bcl-6, in contrast to other effector T cells regulated by Blimp-1 (27). More importantly, constitutive expression of Blimp-1 has an inhibitory effect on Bcl-6 expression and thus represses Tfh cell differentiation (7), indicating that Bcl-6 and Blimp-1 are antagonistic regulators in Tfh cells. The results in B cells might shed light on this mechanism. In plasma cell differentiation, the release of Bcl-6-bound histone deacetylases (HDACs) may increase the histone acetylation levels on the promoter region of Blimp-1, promoting the expression of this gene (28, 29). Thus, HDACs might be the competitive substrate for these two genes. In autoimmune status, Bcl-6 deficiency in lupus-prone mice has been found to impair lupus-like symptoms (30), and increased Bcl-6 has been observed in lupus circulating Tfh-like cells, which is positively correlated with disease activity (31).

Bcl-6 and STAT5

Similar to the Bcl-6-Blimp-1 axis, Bcl-6 and STAT5 also inhibit each other due to their overlapping binding sites in many Tfh cell-related genes, including *Socs2*, *IL7r*, and *Tcf7*. In a mouse study, Bcl-6 has been found to repress both IL-7R and STAT5 expression, as well as inhibiting IL-2-induced STAT5 activation (32). This inhibitory effect on STAT5 by Bcl-6 is due to the abrogation of STAT5 phosphorylation (32). In contrast, signals through IL-2-CD25 activate STAT5 and inhibit Bcl-6 and CXCR5 via inducing Blimp-1 (20, 33, 34), and lack of IL-2R signaling leads to Bcl-6 expression (35). A high concentration of IL-2 has been found to inhibit Bcl-6 expression in polarized Th1 cells, in which the Bcl-6 DNA-binding domain is masked by the T-bet-Bcl-6 complex and normally shows low levels of Bcl-6 expression in response to limited IL-2 (36). However, in response to low IL-2, besides increased Bcl-6 and IL-6R, Th1 cells can also increase the expression of IL-7R, which can repress Tfh-related genes, including *cxc5* and *bcl6* via IL-7-dependent STAT5 activation (37). In addition, Bcl-6 in Tfh cells has been observed to have a decreased level of 5-hydroxymethylcytosine (5hmC), which might explain the markedly high level of Bcl-6 in Tfh cells (32). Conversely, Bcl-6 deficiency results in increased STAT5 signaling and promotes the differentiation of non-Tfh effector T cells. The inhibitory effects of STAT5 have been found to be Blimp-1-independent. In addition, inhibition of IL-2 results in the reduction of Blimp-1 expression (38), indicating that IL-2, STAT5 and Blimp-1 collaboratively inhibit Tfh cell differentiation (39).

STAT3

IL-21 and IL-6/STAT3 are first described to be essential for Th17 cell differentiation (40). Next, STAT3 has found to be critical for Tfh cell differentiation. The evidence come from the fact that reduced IL-21 production is reported in mouse STAT3-deficient

T cells, and only a STAT3 mutation, rather than *Il12RB1*, reduce the frequency of Tfh cells *in vivo* (41). Similarly, in CD4⁺ T cell-conditional STAT3 knockout mice, fewer CXCR5⁺ Tfh cells, as well as defective GCs and reduced IgG and IgM antibody production, have been observed after KLH immunization (42, 43). In another study, the gene expression of *Cxcr5* and *Icos* is shown to be downregulated in STAT3-deficient mice, while the expression of Blimp-1 is increased (44). More importantly, cluster analysis showed that STAT3-deficient Ly6C^{lo} PSGL-1^{hi} T cells in the T cell zone more closely resemble Th1 cells, with a high expression of IFN-induced genes (44). More direct evidence is that STAT3 can form a complex with Ikaros zinc finger transcription factor Aiolos to regulate Bcl-6 expression (45). In a human study, rather than in a mouse system, TGF-beta has been found to provide critical additional signals for STAT3 and STAT4 to initiate Tfh cell differentiation (46), emphasizing the important role of IL-6 in early Tfh cell differentiation, STAT3 deficiency fails to recapitulate the impaired Tfh frequency. However, in this study, STAT1 activity has been found to be required for Bcl-6 induction and initiating Tfh cell differentiation (47). In addition, STAT3 can suppress type 1 IFN induced CD25 expression and can compete with STAT5 to bind to the Bcl6 locus (48). However, it might be difficult to distinguish whether the effects of STAT3 is intrinsic to the Tfh cell or a reflection of diminished capacity for other cell subset differentiation. The forced overexpression of STAT3 in T cell may provide an explanation to this issue, which is still lacking at this moment.

TCF-1 and LEF-1

TCF-1 and LEF-1 belong to the TCF-LEF subfamily and have been well-documented to be necessary for the maturation of double negative T cells to the double positive stage in thymus. In addition, TCF-1 has been reported to restrain mature T cell-mediated Th17 responses via suppressing IL-17 expression (49). TCF-1 and LEF-1 have been reported as critical transcription factors in Tfh cell differentiation by two independent studies published in the same year (50, 51). The loss of either TCF-1 or LEF-1 in mice leads to defects in Tfh cells, and the depletion of both TCF-1 and LEF-1 results in the impairment of Tfh cell differentiation and GC formation. In addition, the important role of LEF-1 has been emphasized by the observation that forced LEF-1 expression promotes the differentiation of Tfh cells (51). In another study, TCF-1 and LEF-1 are revealed to regulate the Bcl-6/Blimp-1 axis. TCF-1 has been identified as a positive regulator for Bcl-6 and it displays negative effects on Blimp-1 via directly binding to the Bcl-6 promoter to form a complex and regulatory region known as intron 3 of *Prdm1* (51). In addition, TCF-1 has been found to upregulate IL-6R expression and inhibit IL-2R expression (51), indicating that TCF-1 might be upstream of STAT3 and STAT5. The exact function of LEF-1 in Tfh cells remains unclear. However, evidence shows that LEF-1 synergistically works with TCF-1 to regulate Tfh cells, and TCF-1 can inhibit LEF-1 expression (51). Furthermore, TCF-1 and LEF-1 have been found to promote early Tfh cell differentiation by maintaining the expression of IL-6Rα and gp130 and enhancing ICOS and Bcl-6 expression (52).

Ascl2

Ascl2 is a basic helix-loop helix (bHLH) transcription factor that has been reported to initiate Tfh cell differentiation via upregulating CXCR5 but not Bcl-6 in T cells *in vitro* (53). In addition, *in vivo*, Ascl2 can promote T cell migration to the border of B cell follicles and can promote Tfh cell differentiation by inhibiting Th1 and Th17 signature genes and upregulating Tfh cell-related genes (53). In other studies, Ascl2 has been shown to be responsible for low CD25 expression on regulatory follicular T cells (Tfr) (54). Ascl2 displays the active chromatin marker trimethylated histone H3 lysine 4 (H3K4me3), which has not been observed in other T cell subsets. In contrast, other Tfh cell-related genes, such as *Bcl-6*, *Maf*, *Batf*, and *Irf4*, are associated with H3K4me3 in all T-cell subsets (55).

C-Maf

c-Maf, a member of the activator protein 1 (AP-1) transcription factor family, has been found to be highly expressed by Th17 and mature Tfh cells compared with CD4⁺ICOS^{hi}CXCR5[−] or CD4⁺ICOS^{lo}CXCR5[−] non-Tfh cells. During Th17 cell differentiation, IL-6 plus TGF- β or IL-21 plus TGF- β can increase the expression of c-Maf, which is ICOS-dependent (56). As mentioned before, Bcl-6 controls the expression of migration genes that are important for the migration of T cells to the follicles. However, the introduction of Bcl-6 cannot alter the production of IL-21 and IL-4, which are the key cytokines produced by Tfh cells. c-Maf has been found to affect the production of IL-21 and CXCR5 (57). In addition, c-Maf and Bcl-6 have been reported to cooperate in the expression of Tfh cell-related genes, such as CXCR4, PD-1, and ICOS (57). The selective loss of c-Maf expression in T cells leads to the inhibition of Tfh cell differentiation in response to vaccinations and bacteria, and it is also critical for high-affinity antibody secretion in vaccinated animals (58). In addition, in Tfh cells, c-Maf has been shown to positively regulate IL-4 production via binding to the conserved noncoding sequence 2 (CNS2) region of the IL-4 locus and via the induction of IRF4 (59–61); however, this effect is c-Maf-independent (61).

Batf

Batf is also a member of the AP-1 family, which lacks transcriptional activation domains (TADs). Batf has been found to be highly expressed by Tfh cells and directly regulates the expression Bcl-6 and c-Maf (62). The expression of Batf has been observed to be regulated by IL-4-STAT6 in Th9 cells and IL-6-STAT3 signaling in M1 mouse myeloid leukemia cells (63–65). In Batf-deficient mouse T cells, the expression of Bcl-6 and c-Maf decreased dramatically, and Bcl-6 alone is not sufficient for Tfh cell differentiation in the absence of Batf (62). In addition, Batf can cooperate with IRF4 along with STAT3 and STAT4 to promote IL-4 production in Tfh cells via binding to the CNS2 region in the IL-4 locus. BATF does not impair IL-4 in Th2 cells but only Tfh cells (61). However, other studies show that the loss of Batf impairs IL-4 production in both Tfh and Th2 cells (66, 67).

IRF4

IRF4 has been well-documented as an important transcription factor in the differentiation of helper T cells and B cells via promoting cell development (68). IRF4 expression in mouse T cells has also been found to promote GC formation by promoting Tfh cell differentiation (69). In IRF4 knockout mice, CD4⁺ T cells in lymph nodes and Peyer's patches failed to express Bcl-6 and Tfh cell-related genes. In addition, the adoptive transfer of wild-type Tfh cells cannot rescue the failed IRF4^{−/−} Tfh cell differentiation (69), indicating a critical role for IRF4 in Tfh cell development. In wild-type mice, IRF4 can interact with JUN and Batf to form a heterotrimer that can bind to AP1-IRF4 complexes and regulate Tfh cell differentiation (69). In another study, IRF4^{−/−} CD4⁺ T cells have impaired STAT3 binding and fail to differentiate into Tfh cells (70). In a recent study, the *Irf4* locus is reported to “sense” the intensity of TCR signaling to determine the *Irf4* expression level. The binding of IRF4 to divergent DNA sequences is regulated by the expression levels of IRF4 and controls Th cell fate determination (71). In Th2 cells, enhancers show a spectrum of occupancy by the Batf-IRF4 complex, which correlates with the sensitivity of gene expression to TCR signal strength (72). The adaptor molecule LAT has been revealed to export the repressor HDAC7 from the nucleus of CD4(+) T cells. The loss of LAT results in impaired TCR signal and the repression of HDAC7 targeted gene *Nur77* and *Irf4* (73). Furthermore, IRF4 has been reported to be induced in a TCR-affinity dependent manner, and it is critical for clonal expansion (74).

In addition, other transcription factors have also been reported to be involved in Tfh cell differentiation. Foxo1, which has been found to negatively regulate Tfh cell differentiation in the early stages of differentiation (75), has also been identified to positively promote Tfh cell differentiation in the late stage of this process (76). However, the molecular mechanism remains unclear. FOXP1 negatively regulates Tfh cell differentiation by directly inhibiting ICOS expression and IL-21 production (77). Kruppel-like factor 2 (KLF2), a transcription factor, has been found to be involved in T cell trafficking, survival and homeostasis. KLF2 deficiency has been linked with increased number of Tfh cells, and forced expression of KLF2 results in reduced Tfh cell differentiation and GC formation (78). KLF2 can negatively control Tfh cell differentiation by inhibiting the homing receptors, such as CXCR5, CCR7, S1PR1 and PSGL1 (79), via induction of negative regulators for Tfh cells, including Blimp-1, T-bet and GATA3 (78).

Other Proteins Regulating Tfh Cell Differentiation E3 Ubiquitin Ligase

Roquin is an RNA binding protein, which has been revealed to play a critical role in innate and adoptive immune systems. The lack of Roquin activity results in numerous autoimmune diseases, such as lupus and inflammatory bowel disease. It is well-known that *sanroque* mice, which have the mutant ROQUIN^{M199R} that promotes Tfh cells, show a spontaneous germinal center (GC) and accumulation of plasma cells (30, 80, 81). The ubiquitin E3 ligase Roquin-1 negatively regulates Tfh cell differentiation by recognizing and directly binding a cis-element in the 3'

untranslated region of ICOS mRNA, thereby repressing ICOS expression (82). The combined loss of Roquin-1 and 2 results in spontaneous Tfh cell and germinal center development (83). Other Tfh-related genes, such as *Il6*, *Irf4*, *Ox40*, (84, 85) and *Ifng* (86), are repressed by Roquin. The loss of the RUNG domain of Roquin has been found to reduce the number of Tfh cells, which might be a result of impaired mTOR signaling (87) and reduced Bcl-6 expression (88). In addition, the E3 ubiquitin ligase Itch has also been reported to regulate Tfh cells by regulating the ubiquitination and degradation of Foxo1 (89), and the effect of Itch has been revealed to be upstream of Bcl-6, which is validated by the fact that forced Bcl-6 in Itch deficient mice can restore Tfh cell differentiation (89). Moreover, the E3 ubiquitin ligase Cullin3 acts as a negative regulator by directly binding to Bcl-6 and regulating the ubiquitination of histone proteins (90). Furthermore, in transplantation, herpesvirus entry mediator/B- and T-lymphocyte attenuator (HVEM/BTLA) signaling pathway has been found to be dispensable for the expansion of Tfh cells and formation of *de novo* host anti-donor isotype-specific antibodies (91).

Notch-1 and -2

The T cell-specific deletion of Notch-1 and Notch-2 results in the reduced number of fully mature Tfh cells and the absence of high-affinity Abs (92). These mature Tfh cells produce low levels of IL-21 and displayed low expression of Bcl-6 and C-Maf. However, the effect of the loss of Notch on Tfh cell differentiation is in an IL-4-independent manner (92). In a recent study, Notch signaling has been identified as an early lineage-determining factor between Tfh and Th2 cell fate (93). In addition, Delta-L 1/4-mediated signals to Tfh cells occur from stroma cells, and follicular dendritic cells are not required (93, 94). Fasnacht et al. (94) shows DLL4 in stromal cells is important for Tfh development. In a previous study, fibroblasts, rather than hematopoietic or endothelial cells, as niche cells, support Notch-2 driven differentiation of marginal-zone B cells, ESAMDCs, and Tfh cells (94).

Surface Molecule Regulation

CXCR5

CXCR5 is a hallmark of Tfh cells that guides T cells to migrate to the B cell zone by binding to CXCL13 that is expressed by follicular dendritic cells (95). CXCL13 is expressed in the follicular mantle zone and not in the endothelial venules and paracortical T cell zone, where ligands for CCR7 exist. Unlike CCR7 ligands, CCL19 and CCL21, CXCL13 controls the segregation between T and B cells, rather than recruiting T cells and B cells to lymph nodes (96). Therefore, these CXCR5^{hi} T cells express a low level of CCR7, which helps these T cells to migrate to GCs (6, 9, 10, 97). Moreover, CXCR5 has been found to help the maintenance of PD-1^{hi} Tfh population in GCs (9). CXCR5-deficient mice have a low GC number and antibody production (95), which shows the important role of CXCR5 in Tfh cell differentiation. In addition to being controlled by Bcl-6, CXCR5 expression is also regulated by nuclear factor of activated T cells (NFAT2) (98).

ICOS/ICOSL

With signals from MHC-antigen-TCR and CD28 stimulation, ICOS expression is induced on activated T cells. Therefore, ICOS is not a reliable marker for Tfh cells not only due to its expression on precursor Tfh cells but also due to its high expression on activated T cells. Signals through ICOS-ICOSL are critical for Tfh cell differentiation, B cell survival and activation, antibody class switching and GC formation (99). In human Tfh cells, ICOS is used as a marker of GC Tfh cells (100). However, ICOS is probably not a reliable marker for GC Tfh cells in mice due to its similar expression in Tfh cells and precursor Tfh cells (101). It has been found that initial DC priming is sufficient to differentiate CXCR5⁺Bcl-6⁺ Tfh cells, but this process depends on consistent ICOS/ICOSL signaling from DCs (102). Further ICOSL signals from B cells are necessary for the complete differentiation and maintenance of GC-Tfh cells (14). ICOS has been found to be capable of regulating the migration of T cells to GCs via the induction of filopodia (17). Signals through ICOS/ICOSL activate phosphoinositide-3 kinase (PI3K) (103), which is also a critical kinase for Tfh cell differentiation via the AKT-mediated inactivation of FOXO (104). In addition, ICOS is able to maintain the Tfh cell phenotype via FOXO1-mediated KLF2 expression (79), and FOXO1 is also inhibited by ICOS-induced mTORc2 (75). ICOS signaling can also affect IL-21 production via c-Maf, thereby regulating Tfh cell differentiation (56). The importance of ICOS in Tfh cells has been demonstrated by a study showing that ICOS-deficient mice have impaired GCs, a reduced level of CXCR5⁺ memory T cells (19, 105), impaired immunoglobulin class switching and low levels of IL-4 when primed *in vivo* and restimulated *in vitro* with a specific antigen (106, 107).

OX40/OX40L

OX40 belongs to the TNFR family and is transiently expressed by T cells during chronic virus infection (108). OX40 has been found to play a critical role in Tfh cell differentiation. Reinforcing OX40 stimulation promotes the expression of Blimp-1 in LCMV-specific T cells and inhibits the differentiation of Tfh cells (108). However, OX40-deficient mice display impaired generation of Tfh cells and GCs (109), indicating that OX40 is important for the Tfh cell differentiation. Indeed, OX40L has been reported to contribute to lupus by promoting Tfh cell responses (110). In addition, TSLP-activated dendritic cells have been found to be able to induce Tfh cell generation via OX40L (111). In addition, OX40 can cooperate with ICOS to amplify Tfh cell development during vaccinia virus infection (112).

Other Important Surface Markers

PD-1, which is usually expressed by exhausted T cells, is highly expressed on Tfh cells. PD-1/PD-Ls signals are generally considered as negative regulatory signals that dephosphorylate TCR signaling, thereby inhibiting activation and cytokine production by T cells (113). PD-1 expressed by Tfh cells is believed to balance the negative regulation from IL-2-mediated STAT5 signaling (114). In addition, Tfr cells also express PD-1, which regulates Tfr cells (115). In a recent report, PD-1 has been found to inhibit follicular T cell recruitment via limiting CXCR3 expression to confine Tfh cell localization in GCs and

increase the stringency of GC affinity selection through PD-1-PD-L1 ligation (116). Cytotoxic T lymphocyte antigen 4 (CTLA-4) is another negative regulator of T cells that has been reported to be expressed by Tfh cells and Tfr cells (117). Tfr and Treg cells regulate Tfh cells via B7-1 and B7-2 binding to CTLA-4. Loss of CTLA-4 in Tfh cells results in the promotion of B cell responses (118). Moreover, mice deficient in the SLAM-associated protein (SAP) show impaired GC formation and defects in T-B cell interaction (22, 119–121). Although SAP deficient T cells can express Tfh markers initially, reduced Tfh cells have been found in GCs from SAP deficient mice (30, 122), suggesting that SAP is required for the generation of functional Tfh cells and the differentiation of Tfh cell contains multiple steps.

Cytokine Regulation

Signals from follicular DCs and the cytokine milieu produced by DCs provide instructions for Tfh cell differentiation. Various cytokines, including IL-6, IL-21, IL-12, IL-23, IL-2, TGF- β , IL-1 β , can regulate Bcl-6, STAT5, and Blimp-1 expression via the JAK-STAT signaling pathway (55). In the early stage of human Tfh cell differentiation, IL-12, IL-23, and TGF- β initiate this process. In addition, other STAT3-activating cytokines, such as IL-1 β and IL-6, support this process in the presence of IL-12, IL-23, and TGF- β . The precursors of Tfh cells share similarities with other Th subsets and can further differentiate into Th1 and Th17 cells dependent on the balance of cytokines (123). Following interactions with B cells, precursor cells can differentiate into Th1-like Tfh cells and Th17-like Tfh cells (123). In addition, some reports have shown that Tfh cells can express

IFN-gamma and IL-4, which provides help for cytokine-driven patterns of immunoglobulin class switching (124). In some autoimmune conditions, such as an experimental autoimmune encephalomyelitis (EAE) model of multiple sclerosis (MS), cells that display a Tfh cell phenotype produce IL-17 (56), and during helminth infection, Tfh cells in lymph nodes produce IL-4 (124–126). These IL-4 producing Tfh cells located in B cell follicles are found to be functionally different from Th2 cells found in peripheral region (124). These IL-4 producing Tfh cells express a low level of GATA3 and no IL-13 (127).

It has been well-established that IL-6-mediated STAT3 activation is critical for IL-21 expression in TCR stimulated mice and human T cells (40, 128). STAT3 can also respond to IL-21 and IL-23. Following cytokine stimulation, STAT3 is phosphorylated by JAK and binds to the Bcl-6 promoter to further promote Bcl-6 transcription (129). In addition, IL-12 has been reported to induce Bcl-6 expression in human T cells via STAT4 activation and has a greater effect on IL-21 production compared to IL-6 and IL-21 (130). In addition, the IL-12-STAT4 pathway can also regulate CXCR5, ICOS, c-Maf, and Batf expression in human T cells (131, 132). TGF- β has been found to enhance the function of STAT3-STAT4 to help T cells to express CXCR5, ICOS, Bcl-6, c-Maf, IL-21, and Batf, as well as to repress the expression of Blimp-1 (42). However, in mice, TGF- β has been reported to have negative regulatory effects on Bcl-6 expression via mir-10a (133). The positive regulation of TGF- β might be restricted to human *in vitro* studies. However, in mice, cytokines and TCR stimulation are insufficient, and the T-B interaction is necessary to generate Tfh cells (134).

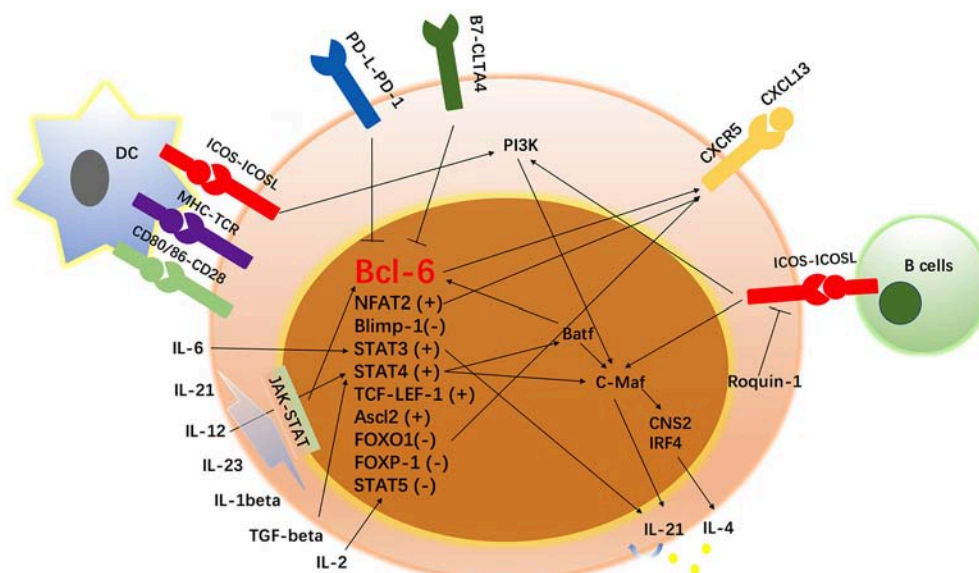


FIGURE 2 | Network of transcription factors, cytokines and surface markers in Tfh cell regulation. In addition to the signals from surface markers, Tfh cells have been found to be regulated by a complex network of transcription factors, including the Bcl-6-Blimp-1 axis, STAT1, STAT3, STAT4, STAT5, B-cell activating transcription factor (Batf), v-maf avian musculoaponeurotic fibrosarcoma oncogene homolog (c-Maf), interferon regulatory factor 4 (IRF4), Achaete-scute homolog 2 (Ascl2), and T-cell-specific transcription factor 1 (TCF-1)-LEF-1, FOXO-1, FOXP-1, and NFAT-2. Since the study of Tfh cells began, some proteins have been identified to participate in the development of Tfh cells. In addition, cytokines such as IL-1 beta, IL-2, IL-6, IL-12, IL-21, IL-23, and TGF- β have been reported to be involved in the differentiation and survival of Tfh cells. “+” means positively regulates Tfh cell differentiation and “-” means negatively regulates Tfh cell development.

Epigenetic Regulations

Epigenetic regulation refers to a modification that will not change the DNA sequence but alters the gene expression through several modifications, such as DNA methylation, histone modification and non-coding RNA-mediated regulations. Increasing evidence has shown the cooperation between epigenetic modifications with transcription factors to determine T cell fate (135).

Unsurprisingly, Tfh cell differentiation is also regulated by epigenetic modifications. DNA methylation refers to silencing gene expression, and demethylation/hydroxymethylation is related to gene reactivation. In Tfh cells, Bcl-6 binding to gene loci has been found to be associated with reduced recruitment of translocation methylcytosine dioxygenase 1 (TET1), which is a hydroxymethyltransferase. Bcl-6 binding is also observed to result in reduced 5-hydroxymethylcytosine (5-hmC) (32), which is a mechanism for DNA demethylation. In addition, in our previous study, we found that IL-21 can increase TET2 enrichment on the promoter region of Bcl-6, which might explain the increased levels of Bcl-6 in lupus T cells (31). In addition, methylated H3K27 has been reported to prevent Tfh-related gene expression, while the H3K27me3 demethylase UTX sustains Tfh cells and antibody production (136). Positive histone modifications have been detected at the *Bcl-6* locus in Tfh cells, but negative marks are present at *Bcl-6* in other Th subsets (137). In addition, positive and negative histone modifications can be detected on *Prdm1* in all Tfh cell populations. These positive and negative histone modifications might provide clues for Tfh cell plasticity. miRNAs, which are non-coding RNAs, regulate gene expression at the posttranscriptional and posttranslational levels. miRNAs silence gene expression by targeting the 3'-untranslated regions of mRNA, causing mRNA cleavage and translational repression. In Tfh cells, the miR-17-92 cluster has been observed to be downregulated, which might contribute to the overexpression of Bcl-6 (138). miR-155 can regulate Tfh cell accumulation in miR-146a-deficient mice, resulting in abnormal Tfh cell accumulation (138). miR-146a can directly targets ICOS and the overexpression of ICOS mediated by the loss of miR-146a results in spontaneous and cell-autonomous Tfh cell accumulation (139). The molecules regulating Tfh cell differentiation are summarized in **Figure 2**.

Increasing evidence has shown the plasticity of Tfh cells, which can be explained by epigenetic regulations. Tfh cells display repressive histone markings (H2K27me3) on *Il4*, *Ifng*, and *Il17a*, while permissive active chromatin H3K4me3 on *Il21* locus (137, 140). Interestingly, the evidence that Tfh cells can produce effector T cell cytokines in response to the polarization cytokines and maintain the ability to produce IL-21 (137), can be explained by the fact that Tfh cells also display detectable H3K4me3 on *Tbx21*, *Gata3*, and *Rorc* locus (137). The positive H3K4me3 has

been observed on the *Bcl6* gene in Tfh cells from an *in vivo* and *ex vivo* system. Other *in vitro* differentiated Th cells also show permissive markers on *Bcl6*, which enables these cells to acquire Tfh cell phenotypes and the capacity to produce IL-21 (137).

CONCLUSIONS

Tfh cell differentiation is regulated by multiple transcription factors, receptors, cytokines, and epigenetic modifications. Unlike other Th cells, mouse Tfh cells are difficult to generate *in vitro* by cytokines and TCR stimulation, possibly reflecting a requirement for T-B cell interactions. ICOS/ICOSL signals might be an underlying explanation for the difficulty mentioned above. In addition, the cytokines driving differentiation in mouse and human systems are different; for example, TGF- β is a negative regulator in mice but a positive regulator in human Tfh cells. Tfh cells are heterogenic populations. Certain Th1, Th2, and Th17-like Tfh cells have been identified in GCs. In addition, Tfr cells have also been reported and regulate Tfh cell homeostasis. In addition, in certain inflammatory sites, such as synovium from rheumatoid arthritis patients, non-classic Tfh-like cells have been identified, which are CXCR5^{low} but have high expression levels of Bcl-6, PD-1, and IL-21. Single-cell mRNA sequencing should facilitate studies aiming at dissecting Tfh cell subset heterogeneity and distribution in tissues and blood. Our understanding of epigenetic regulation of Tfh cells is limited. Due to the development of new technologies, new molecules might be identified in the near future.

AUTHOR CONTRIBUTIONS

HW wrote the manuscript. YD, MZ, JZ, MZ, LL, and GC edited the manuscript. ZH and QL revised the manuscript.

ACKNOWLEDGMENTS

This work was supported by the National Natural Science Foundation of China (No. 81602767, No. 81430074, No. 91442116, No. 81373195, and No. 81771761), the National Basic Research Program of China (No. 2014CB541904), the Natural Science Foundation of Hunan Province (2017JJ3453, 2017SK2042, 2018JJ3756) the National Key Research and Development Program of China (2016YFC0903900), and the Natural Key Clinical Specialty Construction Project of National Health and Family Planning Commission of the People's Republic of China. Many thanks to Prof. Bengt Johansson Lindbom (Lund University, Sweden) for providing valuable suggestions for this review.

REFERENCES

- MacLennan IC, Gulbranson-Judge A, Toellner KM, Casamayor-Palleja M, Chan E, Sze DM, et al. The changing preference of T and B cells for partners as T-dependent antibody responses develop. *Immunol Rev.* (1997) 156:53–66. doi: 10.1111/j.1600-065X.1997.tb00958.x
- Zheng B, Han S, Kelsoe G. T helper cells in murine germinal centers are antigen-specific emigrants that downregulate Thy-1. *J Exp Med.* (1996) 184:1083–91. doi: 10.1084/jem.184.3.1083
- Bowen MB, Butch AW, Parvin CA, Levine A, Nahm MH. Germinal center T cells are distinct helper-inducer T cells. *Hum Immunol.* (1991) 31:67–75. doi: 10.1016/0198-8859(91)90050-J

4. Kelly KA, Bucy RP, Nahm MH. Germinal center T cells exhibit properties of memory helper T cells. *Cell Immunol.* (1995) 163:206–14. doi: 10.1006/cimm.1995.1118
5. Schaerli P, Willmann K, Lang AB, Lipp M, Loetscher P, Moser B. CXC chemokine receptor 5 expression defines follicular homing T cells with B cell helper function. *J Exp Med.* (2000) 192:1553–62. doi: 10.1084/jem.192.11.1553
6. Breitfeld D, Ohl L, Kremmer E, Ellwart J, Sallusto F, Lipp M, et al. Follicular B helper T cells express CXC chemokine receptor 5, localize to B cell follicles, and support immunoglobulin production. *J Exp Med.* (2000) 192:1545–52. doi: 10.1084/jem.192.11.1545
7. Johnston RJ, Poholek AC, DiToro D, Yusuf I, Eto D, Barnett B, et al. Bcl6 and Blimp-1 are reciprocal and antagonistic regulators of T follicular helper cell differentiation. *Science* (2009) 325:1006–10. doi: 10.1126/science.1175870
8. Yu D, Rao S, Tsai LM, Lee SK, He Y, Sutcliffe EL, et al. The transcriptional repressor Bcl-6 directs T follicular helper cell lineage commitment. *Immunity* (2009) 31:457–68. doi: 10.1016/j.immuni.2009.07.002
9. Haynes NM, Allen CD, Lesley R, Ansel KM, Killeen N, Cyster JG. Role of CXCR5 and CCR7 in follicular Th cell positioning and appearance of a programmed cell death gene-1-high germinal center-associated subpopulation. *J Immunol.* (2007) 179:5099–108. doi: 10.4049/jimmunol.179.8.5099
10. Hardtke S, Ohl L, Forster R. Balanced expression of CXCR5 and CCR7 on follicular T helper cells determines their transient positioning to lymph node follicles and is essential for efficient B-cell help. *Blood* (2005) 106:1924–31. doi: 10.1182/blood-2004-11-4494
11. Kim CH, Rott LS, Clark-Lewis I, Campbell DJ, Wu L, Butcher EC. Subspecialization of CXCR5+ T cells: B helper activity is focused in a germinal center-localized subset of CXCR5+ T cells. *J Exp Med.* (2001) 193:1373–81. doi: 10.1084/jem.193.12.1373
12. Luther SA, Bidgol A, Hargreaves DC, Schmidt A, Xu Y, Paniyadi J, et al. Differing activities of homeostatic chemokines CCL19, CCL21, and CXCL12 in lymphocyte and dendritic cell recruitment and lymphoid neogenesis. *J Immunol.* (2002) 169:424–33. doi: 10.4049/jimmunol.169.1.424
13. Hu J, Havenar-Daughton C, Crotty S. Modulation of SAP dependent T:B cell interactions as a strategy to improve vaccination. *Curr Opin Virol.* (2013) 3:363–70. doi: 10.1016/j.coviro.2013.05.015
14. Crotty S. T follicular helper cell differentiation, function, and roles in disease. *Immunity* (2014) 41:529–42. doi: 10.1016/j.immuni.2014.10.004
15. Yu D, Vinuesa CG. The elusive identity of T follicular helper cells. *Trends Immunol.* (2010) 31:377–83. doi: 10.1016/j.it.2010.07.001
16. Walker LS, Gulbranson-Judge A, Flynn S, Brocker T, Raykundalia C, Goodall M, et al. Compromised OX40 function in CD28-deficient mice is linked with failure to develop CXC chemokine receptor 5-positive CD4 cells and germinal centers. *J Exp Med.* (1999) 190:1115–22. doi: 10.1084/jem.190.8.1115
17. Xu H, Li X, Liu D, Li J, Zhang X, Chen X, et al. Follicular T-helper cell recruitment governed by bystander B cells and ICOS-driven motility. *Nature* (2013) 496:523–7. doi: 10.1038/nature12058
18. Liu D, Xu H, Shih C, Wan Z, Ma X, Ma W, et al. T-B-cell entanglement and ICOSL-driven feed-forward regulation of germinal centre reaction. *Nature* (2015) 517:214–8. doi: 10.1038/nature13803
19. Akiba H, Takeda K, Kojima Y, Usui Y, Harada N, Yamazaki T, et al. The role of ICOS in the CXCR5+ follicular B helper T cell maintenance *in vivo*. *J Immunol.* (2005) 175:2340–8. doi: 10.4049/jimmunol.175.4.2340
20. Choi YS, Kageyama R, Eto D, Escobar TC, Johnston RJ, Monticelli L, et al. ICOS receptor instructs T follicular helper cell versus effector cell differentiation via induction of the transcriptional repressor Bcl6. *Immunity* (2011) 34:932–46. doi: 10.1016/j.immuni.2011.03.023
21. Kawamoto S, Tran TH, Maruya M, Suzuki K, Doi Y, Tsutsui Y, et al. The inhibitory receptor PD-1 regulates IgA selection and bacterial composition in the gut. *Science* (2012) 336:485–9. doi: 10.1126/science.1217718
22. Qi H, Cannons JL, Klauschen F, Schwartzberg PL, Germain RN. SAP-controlled T-B cell interactions underlie germinal centre formation. *Nature* (2008) 455:764–9. doi: 10.1038/nature07345
23. Nurieva RI, Chung Y, Martinez GJ, Yang XO, Tanaka S, Matskevitch TD, et al. Bcl6 mediates the development of T follicular helper cells. *Science* (2009) 325:1001–5. doi: 10.1126/science.1176676
24. Poholek AC, Hansen K, Hernandez SG, Eto D, Chande A, Weinstein JS, et al. *In vivo* regulation of Bcl6 and T follicular helper cell development. *J Immunol.* (2010) 185:313–26. doi: 10.4049/jimmunol.0904023
25. Hatzl K, Nance JP, Kroenke MA, Bothwell M, Haddad EK, Melnick A, et al. BCL6 orchestrates Tfh cell differentiation via multiple distinct mechanisms. *J Exp Med.* (2015) 212:539–53. doi: 10.1084/jem.20141380
26. Barish GD, Yu RT, Karunasiri M, Ocampo CB, Dixon J, Benner C, et al. Bcl-6 and NF-kappaB cistromes mediate opposing regulation of the innate immune response. *Genes Dev.* (2010) 24:2760–5. doi: 10.1101/gad.1998010
27. Crotty S, Johnston RJ, Schoenberger SP. Effectors and memories: Bcl-6 and Blimp-1 in T and B lymphocyte differentiation. *Nat Immunol.* (2010) 11:114–20. doi: 10.1038/ni.1837
28. Ochiai K, Muto A, Tanaka H, Takahashi S, Igarashi K. Regulation of the plasma cell transcription factor Blimp-1 gene by Bach2 and Bcl6. *Int Immunol.* (2008) 20:453–60. doi: 10.1093/intimm/dxn005
29. Wu H, Deng Y, Feng Y, Long D, Ma K, Wang X, et al. Epigenetic regulation in B-cell maturation and its dysregulation in autoimmunity. *Cell Mol Immunol.* (2018) 15:676–84. doi: 10.1038/cmi.2017.133
30. Linterman MA, Rigby RJ, Wong RK, Yu D, Brink R, Cannons JL, et al. Follicular helper T cells are required for systemic autoimmunity. *J Exp Med.* (2009) 206:561–76. doi: 10.1084/jem.20081886
31. Huang X, Wu H, Qiu H, Yang H, Deng Y, Zhao M, et al. The expression of Bcl-6 in circulating follicular helper-like T cells positively correlates with the disease activity in systemic lupus erythematosus. *Clin Immunol.* (2016) 173:161–70. doi: 10.1016/j.clim.2016.10.017
32. Liu X, Lu H, Chen T, Nallapareddy KC, Yan X, Tanaka S, et al. Genome-wide analysis identifies Bcl6-controlled regulatory networks during T follicular helper cell differentiation. *Cell Rep.* (2016) 14:1735–47. doi: 10.1016/j.celrep.2016.01.038
33. Gong D, Malek TR. Cytokine-dependent Blimp-1 expression in activated T cells inhibits IL-2 production. *J Immunol.* (2007) 178:242–52. doi: 10.4049/jimmunol.178.1.242
34. Shaffer AL, Lin KI, Kuo TC, Yu X, Hurt EM, Rosenwald A, et al. Blimp-1 orchestrates plasma cell differentiation by extinguishing the mature B cell gene expression program. *Immunity* (2002) 17:51–62. doi: 10.1016/S1074-7613(02)00335-7
35. Pepper M, Pagan AJ, Igyarto BZ, Taylor JJ, Jenkins MK. Opposing signals from the Bcl6 transcription factor and the interleukin-2 receptor generate T helper 1 central and effector memory cells. *Immunity* (2011) 35:583–95. doi: 10.1016/j.immuni.2011.09.009
36. Oestreich KJ, Mohn SE, Weinmann AS. Molecular mechanisms that control the expression and activity of Bcl-6 in TH1 cells to regulate flexibility with a TFH-like gene profile. *Nat Immunol.* (2012) 13:405–11. doi: 10.1038/ni.2242
37. McDonald PW, Read KA, Baker CE, Anderson AE, Powell MD, Ballesteros-Tato A, et al. IL-7 signalling represses Bcl-6 and the TFH gene program. *Nat Commun.* (2016) 7:10285. doi: 10.1038/ncomms10285
38. Nurieva RI, Podd A, Chen Y, Alekseev AM, Yu M, Qi X, et al. STAT5 protein negatively regulates T follicular helper (Tfh) cell generation and function. *J Biol Chem.* (2012) 287:11234–9. doi: 10.1074/jbc.M111.324046
39. Johnston RJ, Choi YS, Diamond JA, Yang JA, Crotty S. STAT5 is a potent negative regulator of TFH cell differentiation. *J Exp Med.* (2012) 209:243–50. doi: 10.1084/jem.20111174
40. Nurieva R, Yang XO, Martinez G, Zhang Y, Panopoulos AD, Ma L, et al. Essential autocrine regulation by IL-21 in the generation of inflammatory T cells. *Nature* (2007) 448:480–3. doi: 10.1038/nature05969
41. Ma CS, Avery DT, Chan A, Batten M, Bustamante J, Boisson-Dupuis S, et al. Functional STAT3 deficiency compromises the generation of human T follicular helper cells. *Blood* (2012) 119:3997–4008. doi: 10.1182/blood-2011-11-392985
42. Nurieva RI, Chung Y, Hwang D, Yang XO, Kang HS, Ma L, et al. Generation of T follicular helper cells is mediated by interleukin-21 but independent of T helper 1, 2, or 17 cell lineages. *Immunity* (2008) 29:138–49. doi: 10.1016/j.immuni.2008.05.009
43. Liu X, Nurieva RI, Dong C. Transcriptional regulation of follicular T-helper (Tfh) cells. *Immunol Rev.* (2013) 252:139–45. doi: 10.1111/imr.12040
44. Edelmann SL, Heissmeyer V. Tfh cell differentiation: missing Stat3 uncovers interferons' interference. *Immunity* (2014) 40:307–9. doi: 10.1016/j.immuni.2014.02.008

45. Read KA, Powell MD, Baker CE, Sreekumar BK, Ringel-Scaia VM, Bachus H, et al. Integrated STAT3 and ikaros zinc finger transcription factor activities regulate Bcl-6 expression in CD4(+) Th cells. *J Immunol.* (2017) 199:2377–87. doi: 10.4049/jimmunol.1700106
46. Schmitt N, Liu Y, Bentebibel SE, Munagala I, Bourdery L, Venuprasad K, et al. The cytokine TGF-beta co-opts signaling via STAT3-STAT4 to promote the differentiation of human TFH cells. *Nat Immunol.* (2014) 15:856–65. doi: 10.1038/ni.2947
47. Choi YS, Eto D, Yang JA, Lao C, Crotty S. Cutting edge: STAT1 is required for IL-6-mediated Bcl6 induction for early follicular helper cell differentiation. *J Immunol.* (2013) 190:3049–53. doi: 10.4049/jimmunol.1203032
48. Ray JP, Marshall HD, Laidlaw BJ, Staron MM, Kaech SM, Craft J. Transcription factor STAT3 and type I interferons are corepressive insulators for differentiation of follicular helper and T helper 1 cells. *Immunity* (2014) 40:367–77. doi: 10.1016/j.immuni.2014.02.005
49. Zhang J, He Z, Sen S, Wang F, Zhang Q, Sun Z. TCF-1 Inhibits IL-17 gene Expression to restrain Th17 immunity in a stage-specific manner. *J Immunol.* (2018) 200:3397–406. doi: 10.4049/jimmunol.1800193
50. Choi YS, Gullicksrud JA, Xing S, Zeng Z, Shan Q, Li F, et al. LEF-1 and TCF-1 orchestrate T(FH) differentiation by regulating differentiation circuits upstream of the transcriptional repressor Bcl6. *Nat Immunol.* (2015) 16:980–90. doi: 10.1038/ni.3226
51. Xu L, Cao Y, Xie Z, Huang Q, Bai Q, Yang X, et al. The transcription factor TCF-1 initiates the differentiation of T(FH) cells during acute viral infection. *Nat Immunol.* (2015) 16:991–9. doi: 10.1038/ni.3229
52. Wu T, Shin HM, Mosseman EA, Ji Y, Huang B, Harly C, et al. TCF1 is required for the T follicular helper cell response to viral infection. *Cell Rep.* (2015) 12:2099–110. doi: 10.1016/j.celrep.2015.08.049
53. Liu X, Chen X, Zhong B, Wang A, Wang X, Chu F, et al. Transcription factor achaete-scute homologue 2 initiates follicular T-helper-cell development. *Nature* (2014) 507:513–8. doi: 10.1038/nature12910
54. Wing JB, Kitagawa Y, Locci M, Hume H, Tay C, Morita T, et al. A distinct subpopulation of CD25(-) T-follicular regulatory cells localizes in the germinal centers. *Proc Natl Acad Sci USA.* (2017) 114:E6400–E9. doi: 10.1073/pnas.1705511114
55. Qiu H, Wu H, Chan V, Lau CS, Lu Q. Transcriptional and epigenetic regulation of follicular T-helper cells and their role in autoimmunity. *Autoimmunity* (2017) 50:71–81. doi: 10.1080/08916934.2017.1284821
56. Bauquet AT, Jin H, Paterson AM, Mitsdoerffer M, Ho IC, Sharpe AH, et al. The costimulatory molecule ICOS regulates the expression of c-Maf and IL-21 in the development of follicular T helper cells and TH-17 cells. *Nat Immunol.* (2009) 10:167–75. doi: 10.1038/ni.1690
57. Kroenke MA, Eto D, Locci M, Cho M, Davidson T, Haddad EK, et al. Bcl6 and Maf cooperate to instruct human follicular helper CD4T cell differentiation. *J Immunol.* (2012) 188:3734–44. doi: 10.4049/jimmunol.1103246
58. Andris F, Denanglaire S, Anciaux M, Hercor M, Hussein H, Leo O. The transcription factor c-Maf promotes the differentiation of follicular helper T cells. *Front Immunol.* (2017) 8:480. doi: 10.3389/fimmu.2017.00480
59. Vijayanand P, Seumois G, Simpson LJ, Abdul-Wajid S, Baumjohann D, Panduro M, et al. Interleukin-4 production by follicular helper T cells requires the conserved Il4 enhancer hypersensitivity site V. *Immunity* (2012) 36:175–87. doi: 10.1016/j.immuni.2011.12.014
60. Harada Y, Tanaka S, Motomura Y, Harada Y, Ohno S, Ohno S, et al. The 3' enhancer CNS2 is a critical regulator of interleukin-4-mediated humoral immunity in follicular helper T cells. *Immunity* (2012) 36:188–200. doi: 10.1016/j.immuni.2012.02.002
61. Sahoo A, Alekseev A, Tanaka K, Obertas L, Lerman B, Haymaker C, et al. Batf is important for IL-4 expression in T follicular helper cells. *Nat Commun.* (2015) 6:7997. doi: 10.1038/ncomms8997
62. Ise W, Kohyama M, Schraml BU, Zhang T, Schwer B, Basu U, et al. The transcription factor BATF controls the global regulators of class-switch recombination in both B cells and T cells. *Nat Immunol.* (2011) 12:536–43. doi: 10.1038/ni.2037
63. Ellyard JJ, Vinuesa CG. A BATF-ling connection between B cells and follicular helper T cells. *Nat Immunol.* (2011) 12:519–20. doi: 10.1038/ni.2042
64. Senga T, Iwamoto T, Humphrey SE, Yokota T, Taparowsky EJ, Hamaguchi M. Stat3-dependent induction of BATF in M1 mouse myeloid leukemia cells. *Oncogene.* (2002) 21:8186–91. doi: 10.1038/sj.onc.1205918
65. Jabeen R, Goswami R, Awe O, Kulkarni A, Nguyen ET, Attenasio A, et al. Th9 cell development requires a BATF-regulated transcriptional network. *J Clin Invest.* (2013) 123:4641–53. doi: 10.1172/JCI69489
66. Bao K, Carr T, Wu J, Barclay W, Jin J, Ciofani M, et al. BATF Modulates the Th2 locus control region and regulates CD4+ T cell fate during antihelminth immunity. *J Immunol.* (2016) 197:4371–81. doi: 10.4049/jimmunol.1601371
67. Betz BC, Jordan-Williams KL, Wang C, Kang SG, Liao J, Logan MR, et al. Batf coordinates multiple aspects of B and T cell function required for normal antibody responses. *J Exp Med.* (2010) 207:933–42. doi: 10.1084/jem.20091548
68. Huber M, Lohoff M. IRF4 at the crossroads of effector T-cell fate decision. *Eur J Immunol.* (2014) 44:1886–95. doi: 10.1002/eji.201344279
69. Bollig N, Brustle A, Kellner K, Ackermann W, Abass E, Raifer H, et al. Transcription factor IRF4 determines germinal center formation through follicular T-helper cell differentiation. *Proc Natl Acad Sci USA.* (2012) 109:8664–9. doi: 10.1073/pnas.1205834109
70. Kwon H, Thierry-Mieg D, Thierry-Mieg J, Kim HP, Oh J, Tunyaplin C, et al. Analysis of interleukin-21-induced Prdm1 gene regulation reveals functional cooperation of STAT3 and IRF4 transcription factors. *Immunity* (2009) 31:941–52. doi: 10.1016/j.immuni.2009.10.008
71. Krishnamoorthy V, Kannanganat S, Maienschein-Cline M, Cook SL, Chen J, Bahroos N, et al. The IRF4 gene regulatory module functions as a read-write integrator to dynamically coordinate T helper cell fate. *Immunity* (2017) 47:481–97 e7. doi: 10.1016/j.immuni.2017.09.001
72. Iwata A, Durai V, Tussiwand R, Briseno CG, Wu X, Grajales-Reyes GE, et al. Quality of TCR signaling determined by different affinities of enhancers for the composite BATF-IRF4 transcription factor complex. *Nat Immunol.* (2017) 18:563–72. doi: 10.1038/ni.3714
73. Myers DR, Lau T, Markegard E, Lim HW, Kasler H, Zhu M, et al. Tonic LAT-HDAC7 signals sustain Nur77 and Irfa expression to tune naive CD4 T cells. *Cell Rep.* (2017) 19:1558–71. doi: 10.1016/j.celrep.2017.04.076
74. Shi W, Man K, Smyth GK, Nutt SL, Kallies A. Whole transcriptome analysis for T cell receptor-affinity and IRF4-regulated clonal expansion of T cells. *Genom Data* (2014) 2:396–8. doi: 10.1016/j.gdata.2014.10.019
75. Zeng H, Cohen S, Guy C, Shrestha S, Neale G, Brown SA, et al. mTORC1 and mTORC2 kinase signaling and glucose metabolism drive follicular helper T cell differentiation. *Immunity* (2016) 45:540–54. doi: 10.1016/j.immuni.2016.08.017
76. Stone EL, Pepper M, Katayama CD, Kerdiles YM, Lai CY, Emslie E, et al. ICOS coreceptor signaling inactivates the transcription factor FOXO1 to promote Tfh cell differentiation. *Immunity* (2015) 42:239–51. doi: 10.1016/j.immuni.2015.01.017
77. Wang H, Geng J, Wen X, Bi E, Kossenkova AV, Wolf AI, et al. The transcription factor Foxp1 is a critical negative regulator of the differentiation of follicular helper T cells. *Nat Immunol.* (2014) 15:667–75. doi: 10.1038/ni.2890
78. Lee JY, Skon CN, Lee YJ, Oh S, Taylor JJ, Malhotra D, et al. The transcription factor KLF2 restrains CD4(+) T follicular helper cell differentiation. *Immunity* (2015) 42:252–64. doi: 10.1016/j.immuni.2015.01.013
79. Weber JP, Fuhrmann F, Feist RK, Lahmann A, Al Baz MS, Gentz LJ, et al. ICOS maintains the T follicular helper cell phenotype by down-regulating Kruppel-like factor 2. *J Exp Med.* (2015) 212:217–33. doi: 10.1084/jem.20141432
80. Vinuesa CG, Cook MC, Angelucci C, Athanasopoulos V, Rui L, Hill KM, et al. A RING-type ubiquitin ligase family member required to repress follicular helper T cells and autoimmunity. *Nature* (2005) 435:452–8. doi: 10.1038/nature03555
81. Bertossi A, Aichinger M, Sansonetti P, Lech M, Neff F, Pal M, et al. Loss of Roquin induces early death and immune deregulation but not autoimmunity. *J Exp Med.* (2011) 208:1749–56. doi: 10.1084/jem.20110578
82. Heissmeyer V, Vogel KU. Molecular control of Tfh-cell differentiation by Roquin family proteins. *Immunol Rev.* (2013) 253:273–89. doi: 10.1111/imr.12056
83. Pratama A, Ramiscal RR, Silva DG, Das SK, Athanasopoulos V, Fitch J, et al. Roquin-2 shares functions with its paralog Roquin-1 in the repression

- of mRNAs controlling T follicular helper cells and systemic inflammation. *Immunity* (2013) 38:669–80. doi: 10.1016/j.immuni.2013.01.011
84. Tan D, Zhou M, Kiledjian M, Tong L. The ROQ domain of Roquin recognizes mRNA constitutive-decay element and double-stranded RNA. *Nat Struct Mol Biol.* (2014) 21:679–85. doi: 10.1038/nsmb.2857
 85. Vogel KU, Edelmann SL, Jeltsch KM, Bertossi A, Heger K, Heinz GA, et al. Roquin paralogs 1 and 2 redundantly repress the Icos and Ox40 costimulator mRNAs and control follicular helper T cell differentiation. *Immunity* (2013) 38:655–68. doi: 10.1016/j.immuni.2012.12.004
 86. Lee SK, Silva DG, Martin JL, Pratama A, Hu X, Chang PP, et al. Interferon-gamma excess leads to pathogenic accumulation of follicular helper T cells and germinal centers. *Immunity* (2012) 37:880–92. doi: 10.1016/j.immuni.2012.10.010
 87. Ramiscal RR, Parish IA, Lee-Young RS, Babon JJ, Blagih J, Pratama A, et al. Attenuation of AMPK signaling by ROQUIN promotes T follicular helper cell formation. *Elife* (2015) 4:e08698. doi: 10.7554/eLife.08698
 88. Ding Y, Li J, Yang P, Luo B, Wu Q, Zajac AJ, et al. Interleukin-21 promotes germinal center reaction by skewing the follicular regulatory T cell to follicular helper T cell balance in autoimmune BXD2 mice. *Arthritis Rheumatol.* (2014) 66:2601–12. doi: 10.1002/art.38735
 89. Xiao N, Eto D, Elly C, Peng G, Crotty S, Liu YC. The E3 ubiquitin ligase Itch is required for the differentiation of follicular helper T cells. *Nat Immunol.* (2014) 15:657–66. doi: 10.1038/ni.2912
 90. Mathew R, Mao AP, Chiang AH, Bertozzi-Villa C, Bunker JJ, Scanlon ST, et al. A negative feedback loop mediated by the Bcl6-cullin 3 complex limits Tfh cell differentiation. *J Exp Med.* (2014) 211:1137–51. doi: 10.1084/jem.20132267
 91. Rodriguez-Barbosa JI, Fernandez-Renedo C, Moral AMB, Buhler L, Del Rio ML. T follicular helper expansion and humoral-mediated rejection are independent of the HVEM/BTLA pathway. *Cell Mol Immunol.* (2017) 14:497–510. doi: 10.1038/cmi.2015.101
 92. Auderset F, Schuster S, Fasnacht N, Coutaz M, Charmoy M, Koch U, et al. Notch signaling regulates follicular helper T cell differentiation. *J Immunol.* (2013) 191:2344–50. doi: 10.4049/jimmunol.1300643
 93. Dell'Aringa M, Reinhardt RL. Notch signaling represents an important checkpoint between follicular T-helper and canonical T-helper 2 cell fate. *Mucosal Immunol.* (2018) 11:1079–91. doi: 10.1038/s41385-018-0012-9
 94. Fasnacht N, Huang HY, Koch U, Favre S, Auderset F, Chai Q, et al. Specific fibroblastic niches in secondary lymphoid organs orchestrate distinct Notch-regulated immune responses. *J Exp Med.* (2014) 211:2265–79. doi: 10.1084/jem.20132528
 95. Ansel KM, McHeyzer-Williams LJ, Ngo VN, McHeyzer-Williams MG, Cyster JG. *In vivo*-activated CD4 T cells upregulate CXCR5 chemokine receptor 5 and reprogram their response to lymphoid chemokines. *J Exp Med.* (1999) 190:1123–34. doi: 10.1084/jem.190.8.1123
 96. Ngo VN, Korner H, Gunn MD, Schmidt KN, Riminton DS, Cooper MD, et al. Lymphotoxin alpha/beta and tumor necrosis factor are required for stromal cell expression of homing chemokines in B and T cell areas of the spleen. *J Exp Med.* (1999) 189:403–12. doi: 10.1084/jem.189.2.403
 97. Arnold CN, Campbell DJ, Lipp M, Butcher EC. The germinal center response is impaired in the absence of T cell-expressed CXCR5. *Eur J Immunol.* (2007) 37:100–9. doi: 10.1002/eji.200636486
 98. Vaeth M, Muller G, Stauss D, Dietz L, Klein-Hessling S, Serfling E, et al. Follicular regulatory T cells control humoral autoimmunity via NFAT2-regulated CXCR5 expression. *J Exp Med.* (2014) 211:545–61. doi: 10.1084/jem.20130604
 99. Vinuesa CG, Tangye SG, Moser B, Mackay CR. Follicular B helper T cells in antibody responses and autoimmunity. *Nat Rev Immunol.* (2005) 5:853–65. doi: 10.1038/nri1714
 100. Benteibibel SE, Schmitt N, Banchereau J, Ueno H. Human tonsil B-cell lymphoma 6 (BCL6)-expressing CD4+ T-cell subset specialized for B-cell help outside germinal centers. *Proc Natl Acad Sci USA.* (2011) 108:E488–97. doi: 10.1073/pnas.1100898108
 101. Shulman Z, Gitlin AD, Targ S, Jankovic M, Pasqual G, Nussenzweig MC, et al. T follicular helper cell dynamics in germinal centers. *Science* (2013) 341:673–7. doi: 10.1126/science.1241680
 102. Qi H, Liu D, Ma W, Wang Y, Yan H. Bcl-6 controlled TFH polarization and memory: the known unknowns. *Curr Opin Immunol.* (2014) 28:34–41. doi: 10.1016/j.coi.2014.01.016
 103. Rolf J, Fairfax K, Turner M. Signaling pathways in T follicular helper cells. *J Immunol.* (2010) 184:6563–8. doi: 10.4049/jimmunol.1000202
 104. Calnan DR, Brunet A. The FoxO code. *Oncogene* (2008) 27:2276–88. doi: 10.1038/onc.2008.21
 105. Bossaller L, Burger J, Draeger R, Grimbacher B, Knoth R, Plebani A, et al. ICOS deficiency is associated with a severe reduction of CXCR5+CD4 germinal center Th cells. *J Immunol.* (2006) 177:4927–32. doi: 10.4049/jimmunol.177.7.4927
 106. Tafuri A, Shahinian A, Bladt F, Yoshinaga SK, Jordana M, Wakeham A, et al. ICOS is essential for effective T-helper-cell responses. *Nature* (2001) 409:105–9. doi: 10.1038/35051113
 107. Dong C, Temann UA, Flavell RA. Cutting edge: critical role of inducible costimulator in germinal center reactions. *J Immunol.* (2001) 166:3659–62. doi: 10.4049/jimmunol.166.6.3659
 108. Boettler T, Choi YS, Salek-Ardakani S, Cheng Y, Moeckel F, Croft M, et al. Exogenous OX40 stimulation during lymphocytic choriomeningitis virus infection impairs follicular Th cell differentiation and diverts CD4 T cells into the effector lineage by upregulating Blimp-1. *J Immunol.* (2013) 191:5026–35. doi: 10.4049/jimmunol.1300013
 109. Boettler T, Moeckel F, Cheng Y, Heeg M, Salek-Ardakani S, Crotty S, et al. OX40 facilitates control of a persistent virus infection. *PLoS Pathog.* (2012) 8:e1002913. doi: 10.1371/journal.ppat.1002913
 110. Jacquemin C, Schmitt N, Contin-Bordes C, Liu Y, Narayanan P, Seneschal J, et al. OX40 ligand contributes to human lupus pathogenesis by promoting T follicular helper response. *Immunity* (2015) 42:1159–70. doi: 10.1016/j.immuni.2015.05.012
 111. Pattarini L, Trichot C, Bogiatzi S, Grandclaudon M, Meller S, Keuylian Z, et al. TSLP-activated dendritic cells induce human T follicular helper cell differentiation through OX40-ligand. *J Exp Med.* (2017) 214:1529–46. doi: 10.1084/jem.20150402
 112. Tahilian V, Hutchinson TE, Abboud G, Croft M, Salek-Ardakani S. OX40 cooperates with ICOS to amplify follicular Th cell development and germinal center reactions during infection. *J Immunol.* (2017) 198:218–28. doi: 10.4049/jimmunol.1601356
 113. Bardhan K, Anagnostou T, Boussiotis VA. The PD1:PD-L1/2 pathway from discovery to clinical implementation. *Front Immunol.* (2016) 7:550. doi: 10.3389/fimmu.2016.00550
 114. Jogdand GM, Mohanty S, Devadas S. Regulators of Tfh cell differentiation. *Front Immunol.* (2016) 7:520. doi: 10.3389/fimmu.2016.00520
 115. Sage PT, Francisco LM, Carman CV, Sharpe AH. The receptor PD-1 controls follicular regulatory T cells in the lymph nodes and blood. *Nat Immunol.* (2013) 14:152–61. doi: 10.1038/ni.2496
 116. Shi J, Hou S, Fang Q, Liu X, Liu X, Qi H. PD-1 controls follicular T helper cell positioning and function. *Immunity* (2018) 49:264–74.e4. doi: 10.1016/j.immuni.2018.06.012
 117. Linterman MA, Pierson W, Lee SK, Kallies A, Kawamoto S, Rayner TF, et al. Foxp3+ follicular regulatory T cells control the germinal center response. *Nat Med.* (2011) 17:975–82. doi: 10.1038/nm.2425
 118. Wing JB, Ise W, Kurosaki T, Sakaguchi S. Regulatory T cells control antigen-specific expansion of Tfh cell number and humoral immune responses via the coreceptor CTLA-4. *Immunity* (2014) 41:1013–25. doi: 10.1016/j.immuni.2014.12.006
 119. Cannons JL, Qi H, Lu KT, Dutta M, Gomez-Rodriguez J, Cheng J, et al. Optimal germinal center responses require a multistage T cell:B cell adhesion process involving integrins, SLAM-associated protein, and CD84. *Immunity* (2010) 32:253–65. doi: 10.1016/j.immuni.2010.01.010
 120. Crotty S, Kersh EN, Cannons J, Schwartzberg PL, Ahmed R. SAP is required for generating long-term humoral immunity. *Nature* (2003) 421:282–7. doi: 10.1038/nature01318
 121. Czar MJ, Kersh EN, Mijares LA, Lanier G, Lewis J, Yap G, et al. Altered lymphocyte responses and cytokine production in mice deficient in the X-linked lymphoproliferative disease gene SH2D1A/DSHP/SAP. *Proc Natl Acad Sci USA.* (2001) 98:7449–54. doi: 10.1073/pnas.131193098
 122. Yusuf I, Kageyama R, Monticelli L, Johnston RJ, Ditoro D, Hansen K, et al. Germinal center T follicular helper cell IL-4 production is dependent on

- signaling lymphocytic activation molecule receptor (CD150). *J Immunol.* (2010) 185:190–202. doi: 10.4049/jimmunol.0903505
123. Ueno H, Banchereau J, Vinuesa CG. Pathophysiology of T follicular helper cells in humans and mice. *Nat Immunol.* (2015) 16:142–52. doi: 10.1038/ni.3054
 124. Reinhardt RL, Liang HE, Locksley RM. Cytokine-secreting follicular T cells shape the antibody repertoire. *Nat Immunol.* (2009) 10:385–93. doi: 10.1038/ni.1715
 125. King IL, Mohrs M. IL-4-producing CD4⁺ T cells in reactive lymph nodes during helminth infection are T follicular helper cells. *J Exp Med.* (2009) 206:1001–7. doi: 10.1084/jem.20090313
 126. Glatman Zaretsky A, Taylor JJ, King IL, Marshall FA, Mohrs M, Pearce EJ. T follicular helper cells differentiate from Th2 cells in response to helminth antigens. *J Exp Med.* (2009) 206:991–9. doi: 10.1084/jem.20090303
 127. Liang HE, Reinhardt RL, Bando JK, Sullivan BM, Ho IC, Locksley RM. Divergent expression patterns of IL-4 and IL-13 define unique functions in allergic immunity. *Nat Immunol.* (2011) 13:58–66. doi: 10.1038/ni.2182
 128. Yang Y, Ochando J, Yopp A, Bromberg JS, Ding Y. IL-6 plays a unique role in initiating c-Maf expression during early stage of CD4 T cell activation. *J Immunol.* (2005) 174:2720–9. doi: 10.4049/jimmunol.174.5.2720
 129. Eto D, Lao C, DiToro D, Barnett B, Escobar TC, Kageyama R, et al. IL-21 and IL-6 are critical for different aspects of B cell immunity and redundantly induce optimal follicular helper CD4 T cell (Tfh) differentiation. *PLoS ONE* (2011) 6:e17739. doi: 10.1371/journal.pone.0017739
 130. Ma CS, Suryani S, Avery DT, Chan A, Nanan R, Santner-Nanan B, et al. Early commitment of naive human CD4⁺ T cells to the T follicular helper (T(FH)) cell lineage is induced by IL-12. *Immunol Cell Biol.* (2009) 87:590–600. doi: 10.1038/icb.2009.64
 131. Nakayamada S, Kanno Y, Takahashi H, Jankovic D, Lu KT, Johnson TA, et al. Early Th1 cell differentiation is marked by a Tfh cell-like transition. *Immunity* (2011) 35:919–31. doi: 10.1016/j.immuni.2011.11.012
 132. Wei L, Vahedi G, Sun HW, Watford WT, Takatori H, Ramos HL, et al. Discrete roles of STAT4 and STAT6 transcription factors in tuning epigenetic modifications and transcription during T helper cell differentiation. *Immunity* (2010) 32:840–51. doi: 10.1016/j.immuni.2010.06.003
 133. McCarron MJ, Marie JC. TGF-beta prevents T follicular helper cell accumulation and B cell autoreactivity. *J Clin Invest.* (2014) 124:4375–86. doi: 10.1172/JCI76179
 134. Kolenbrander A, Grewe B, Nemazee D, Uberla K, Temchura V. Generation of T follicular helper cells *in vitro*: requirement for B-cell receptor cross-linking and cognate B- and T-cell interaction. *Immunology* (2018) 153:214–24. doi: 10.1111/imm.12834
 135. Kitagawa Y, Wing JB, Sakaguchi S. Transcriptional and epigenetic control of regulatory T cell development. *Prog Mol Biol Transl Sci.* (2015) 136:1–33. doi: 10.1016/bs.pmbts.2015.07.011
 136. Cook KD, Shpargel KB, Starmer J, Whitfield-Larry F, Conley B, Allard DE, et al. T Follicular Helper cell-dependent clearance of a persistent virus infection requires T cell expression of the histone demethylase UTX. *Immunity* (2015) 43:703–14. doi: 10.1016/j.immuni.2015.09.002
 137. Lu KT, Kanno Y, Cannons JL, Handon R, Bible P, Elkhouloun AG, et al. Functional and epigenetic studies reveal multistep differentiation and plasticity of *in vitro*-generated and *in vivo*-derived follicular T helper cells. *Immunity* (2011) 35:622–32. doi: 10.1016/j.immuni.2011.07.015
 138. Kang SG, Liu WH, Lu P, Jin HY, Lim HW, Shepherd J, et al. MicroRNAs of the miR-17 approximately 92 family are critical regulators of T(FH) differentiation. *Nat Immunol.* (2013) 14:849–57. doi: 10.1038/ni.2648
 139. Pratama A, Srivastava M, Williams NJ, Papa I, Lee SK, Dinh XT, et al. MicroRNA-146a regulates ICOS-ICOSL signalling to limit accumulation of T follicular helper cells and germinal centres. *Nat Commun.* (2015) 6:6436. doi: 10.1038/ncomms7436
 140. Wei G, Wei L, Zhu J, Zang C, Hu-Li J, Yao Z, et al. Global mapping of H3K4me3 and H3K27me3 reveals specificity and plasticity in lineage fate determination of differentiating CD4⁺ T cells. *Immunity* (2009) 30:155–67. doi: 10.1016/j.immuni.2008.12.009

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2018 Wu, Deng, Zhao, Zhang, Zheng, Chen, Li, He and Lu. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



Regulation of the Germinal Center Response

Marisa Stebegg^{1†}, Saumya D. Kumar^{2,3†}, Alyssa Silva-Cayetano^{1†}, Valter R. Fonseca^{2,4}, Michelle A. Linterman^{1*‡} and Luis Graca^{2,3*‡}

¹ Babraham Institute, Cambridge, United Kingdom, ² Instituto de Medicina Molecular, Faculdade de Medicina, Universidade de Lisboa, Lisbon, Portugal, ³ Instituto Gulbenkian de Ciência, Oeiras, Portugal, ⁴ Centro Hospitalar Lisboa Norte-Hospital de Santa Maria, Lisbon, Portugal

OPEN ACCESS

Edited by:

Shahram Salek-Ardakani,
Pfizer, United States

Reviewed by:

Betty Diamond,
Feinstein Institute for Medical
Research, United States
Oliver Bannard,
University of Oxford, United Kingdom

*Correspondence:

Luis Graca
lgraca@medicina.ulisboa.pt
Michelle A. Linterman
michelle.linterman@babraham.ac.uk

[†]These authors have contributed
equally to this work

[‡]These authors share senior
authorship

Specialty section:

This article was submitted to
T Cell Biology,
a section of the journal
Frontiers in Immunology

Received: 31 July 2018

Accepted: 05 October 2018

Published: 25 October 2018

Citation:

Stebegg M, Kumar SD,
Silva-Cayetano A, Fonseca VR,
Linterman MA and Graca L (2018)
Regulation of the Germinal Center
Response. *Front. Immunol.* 9:2469.
doi: 10.3389/fimmu.2018.02469

The germinal center (GC) is a specialized microstructure that forms in secondary lymphoid tissues, producing long-lived antibody secreting plasma cells and memory B cells, which can provide protection against reinfection. Within the GC, B cells undergo somatic mutation of the genes encoding their B cell receptors which, following successful selection, can lead to the emergence of B cell clones that bind antigen with high affinity. However, this mutation process can also be dangerous, as it can create autoreactive clones that can cause autoimmunity. Because of this, regulation of GC reactions is critical to ensure high affinity antibody production and to enforce self-tolerance by avoiding emergence of autoreactive B cell clones. A productive GC response requires the collaboration of multiple cell types. The stromal cell network orchestrates GC cell dynamics by controlling antigen delivery and cell trafficking. T follicular helper (Tfh) cells provide specialized help to GC B cells through cognate T-B cell interactions while Foxp3⁺ T follicular regulatory (Tfr) cells are key mediators of GC regulation. However, regulation of GC responses is not a simple outcome of Tfh/Tfr balance, but also involves the contribution of other cell types to modulate the GC microenvironment and to avoid autoimmunity. Thus, the regulation of the GC is complex, and occurs at multiple levels. In this review we outline recent developments in the biology of cell subsets involved in the regulation of GC reactions, in both secondary lymphoid tissues, and Peyer's patches (PPs). We discuss the mechanisms which enable the generation of potent protective humoral immunity whilst GC-derived autoimmunity is avoided.

Keywords: germinal center (GC), Tfr cell, Tfh cell, immunoregulation, humoral responses

Interactions between T and B cells are critical for the development of most humoral immune responses; these can be protective in response to vaccination or infection, or deleterious, when driving autoimmunity, allergy, or transplant rejection. Long-lived T-dependent humoral immunity is derived from specialized microanatomical structures known as germinal centers (GCs), that form in secondary lymphoid organs, such as the spleen and lymph nodes, upon infection, or immunization with a T-cell dependent antigen (**Figure 1**) (1). Ectopic GCs can also appear in non-lymphoid tissue in multiple inflammatory states including autoimmune disease, cancer, and during infection (2). Within GCs, B cells undergo somatic hypermutation (SHM) of the genes encoding their B cell receptor (BCR). Because this mutational process is random, mutated B cells require selection to ensure that only B cells bearing a BCR with an improved affinity for antigen differentiate into long-lived antibody secreting plasma cells and memory B cells (3). Therefore, tight regulation

of GCs is critical to ensure that a potent immune response against foreign antigen can occur without cross reactivity against self-antigens.

For B cells to participate in the GC response, they first need to recognize their cognate antigen via their BCR. B cells are able to directly bind soluble antigen or bind antigen presented on the surface of follicular dendritic cells (FDCs), macrophages or dendritic cells (4–7). Once activated by antigen encounter, B cells upregulate the chemokine receptor CCR7, which facilitates the migration of B cells via a chemokine gradient toward the CCR7 ligands CCL19 and CCL21 expressed in the T cell zone (8). At the interface between the B cell follicle and the T cell zone (T:B border), B cells present fragments of peptide antigen on major histocompatibility complex (MHC)-Class II to CD4⁺ helper T cells that provide them with survival and co-stimulatory signals (8, 9). B cells will then divide at the perimeter of the follicle and will either initiate the GC response or differentiate into short-lived extrafollicular plasma cells or memory B cells (10–12). Extrafollicular plasma cells produce the first wave of antibodies before undergoing apoptosis within a few days, providing an initial burst of antibodies that are essential for early control of infection while the GC response is established (13).

After cognate interactions with CD4⁺ T cells, activated B cells will migrate to the center of the follicle to seed the GC response (14). These GC B cell precursors begin to rapidly divide and undergo clonal expansion during which the GC is divided into two distinct compartments known as the dark zone (DZ) and the light zone (LZ; **Figure 1**) (15). The DZ contains the rapidly dividing B cells known as centroblasts, which undergo SHM (16–18). Centroblasts express the chemokine receptor CXCR4 whose ligand, CXCL12, is produced by stromal cells in the DZ (CXCL12-expressing reticular cells, CRCs). This chemokine localizes the centroblasts within the DZ, thereby generating GC polarity (16, 19). Once GC B cells have undergone SHM in the DZ, they downregulate CXCR4 and migrate to the LZ, to receive positive selection signals. The LZ is rich in FDCs that produce CXCL13, which attracts GC B cells that exit the DZ as centrocytes, through their expression of CXCR5 (15, 17, 18). The LZ also contains Tfh and Tfr cells that are important for the successful and regulated continuation of the GC response (3). FDCs and Tfh cells are critical for the positive selection of centrocytes, while Tfr cells are thought to regulate the output of the GC response (3). Together, these processes culminate in the emergence of long-lived antibody secreting plasma cells and memory B cells whose BCRs bind antigen with high affinity. These effector cells are able to provide protection against subsequent infection, in some cases providing life-long immunity against particular pathogens.

DC-INITIATED TFH CELL DEVELOPMENT IS ESSENTIAL FOR THE GC RESPONSE

Tfh cells are unique in their ability to support GC reactions. Tfh differentiation is a multistage process (20). First, naïve CD4⁺ T cells are primed by dendritic cells (DCs). During these interactions, T cells require two signals to be activated: first binding of the T cell receptor (TCR) to peptide:MHC and

secondly, a co-stimulatory signal through ligation of the receptor CD28 by its ligands CD80/86 which are expressed on the surface of DCs. During this T:DC interaction, T cells also integrate signals from multiple cytokines that skew their differentiation toward a Tfh cell fate. Here, Tfh cell precursors (pre-Tfh cells) upregulate Bcl6 and CXCR5, and downregulate CCR7, leading to migration of activated T cells toward the T:B border. Here, SAP-dependent interactions with activated B cells enable full Tfh cell differentiation.

It is now clear that specific subsets of DCs can support the initial steps of Tfh differentiation (21). Although it appears that this is not a “one DC fits all responses” rule as different types of immune stimuli trigger different DC populations to support Tfh cell differentiation. Adjuvants that trigger Toll-like receptor (TLR)-9 enable monocyte derived DCs to induce Tfh differentiation (22). In Th2 skewed responses, CD8a⁺ conventional dendritic cells (cDCs) are capable of inducing Tfh cell differentiation through higher expression of ICOSL and OX40L co-stimulatory signals compared to CD8a⁺ DCs in both mice and humans (23, 24). Similarly, CD11b⁺ cDC (cDC2, which are CD8a⁺) cells are both necessary and sufficient for Tfh induction following intranasal immunization (25). These cDC2 have a phenotype consistent with location at the T:B border. In contrast, CD301b⁺ DCs are thought to limit effective Tfh differentiation and antibody responses following immunization with type 2 adjuvants (26), through expression of the inhibitory costimulatory ligand PD-L1 (26). Taken together, priming of naïve CD4⁺ T cells by DCs is essential for the first step in Tfh cell differentiation, but multiple DC types are capable of doing the job.

REGULATION OF THE GC RESPONSE BY CHEMOKINES AND THE STROMAL CELL NETWORK

Chemokines and Immune Cell Migration

The chemokine system coordinates the migration and positioning of immune cells within secondary lymphoid organs (27). Chemokines are typically secreted chemotactic cytokines that constitute a family of more than 40 small proteins with a molecular weight of 7–12 kDa (28). Chemokines are able to mediate the migration and positioning of immune cells by engaging G protein-coupled receptors (GPCRs), expressed on the surface of all immune cells, with high affinity (29). Various lymphoid and non-lymphoid cells are able to express chemokines (29), however the expression of chemokines by mesenchymal stromal cells is critical for guiding lymphocytes and dendritic cells (DCs) to secondary lymphoid organs (SLOs) during the initiation of the immune response (27). Within the SLOs, different types of stromal cells play specific roles to facilitate the localization of hematopoietic cells (**Figure 2**). In the T cell zone, fibroblastic reticular cells (FRCs) orchestrate the migration, localization and survival of DCs, T cells and B cells by producing CCL19, CCL21, and CXCL12 (30–32). The CCL19/21 produced by FRCs enables localization of both CD4⁺ T cells and DCs to the T cell zone, via CCR7-mediated migration, bringing

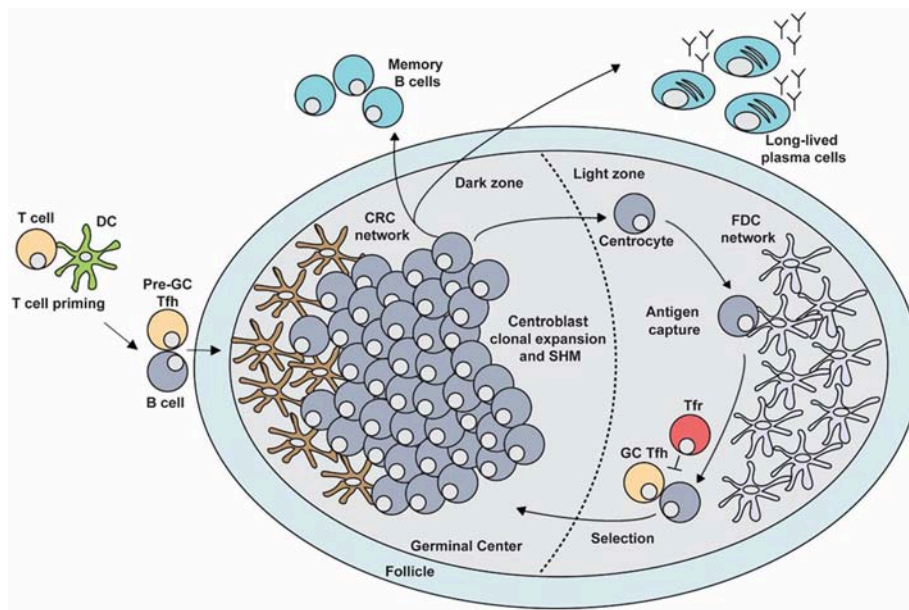


FIGURE 1 | The germinal center (GC) response. The GC is a specialized microenvironment formed within the B cell follicles of secondary lymphoid tissues upon infection or immunization. The GC is divided into two distinct compartments. The dark zone (DZ) that contains a network of CXCL12-producing reticular cells (CRCs) and is the site of GC B cell proliferation and somatic hypermutation (SHM). Centroblasts then follow a CXCL13 gradient to enter the light zone (LZ) as centrocytes through their expression of CXCR5. In the LZ, centrocytes capture antigen presented on follicular dendritic cells (FDCs) which they internalize, process and subsequently present to T follicular helper (Tfh) cells in order to undergo selection. This process is regulated by T follicular regulatory (Tfr) cells which are also present in the LZ. Upon receiving survival signals from Tfh cells, centrocytes re-enter the DZ for further rounds of proliferation and SHM after which they exit the GC as memory B cells or high-affinity antibody-secreting plasma cells.

these rare cells together to facilitate T cell priming and activation (33–35). In the B cell follicles there are two types of stromal cells: follicular dendritic cells (FDCs) that produce CXCL13 and CXCL12-producing reticular cells (CRCs) which promote the localization of B cells during the germinal center response (19, 36, 37). FRCs at the boundary of the T cell zone and follicle produce B cell-activating factor (BAFF) to maintain the primary follicle structure (30). Antigen encounter by naïve B cells is facilitated by CXCR5-mediated migration toward the CXCL13-rich follicles (38–40). This localizes them close to the subcapsular sinus (SCS) where small soluble antigens are drained and can directly trigger B cell activation (38–40). Alternatively, antigens drained through the SCS can be captured by follicular FDCs which typically recognize antigen bound by antibody and/or complement, known as immune complexes (ICs) (41). FDCs are able to retain antigen on their surface for prolonged periods of time, allowing B cells to scan the follicular FDC network for cognate antigen to trigger activation (42). In addition, the SCS facilitates the movement of lymph fluid and is lined by marginal reticular cells (MRCs), which are FDC precursors and provide structural support (43). The distribution of these stromal cells in specific areas of the SLOs facilitates the continuous circulation and subsequent activation of lymphocytes that enter the LNs and Peyer's patches (PPs) through high endothelial venules (HEV) (44). Together, the stromal cell network provides the structural and chemotactic support required for GC initiation and maintenance.

Stromal Cells and Chemokine Gradients Regulate GC Initiation

The initiation of the GC requires both $CD4^+$ T cells and B cells to be activated by cognate antigen. The initial encounter and activation of lymphocytes by antigen is facilitated by stromal cell networks, as described above. Once both $CD4^+$ T cells and B cells are activated by antigen, they must migrate toward the T:B interface to undergo cognate interactions that ultimately lead to GC formation (27). Activated $CD4^+$ T cells begin to downregulate CCR7 and upregulate CXCR5 which allows them to move away from the CCL19/21-rich T cell zone and toward the interfollicular region (45–47). Simultaneously, activated B cells upregulate CCR7 while maintaining CXCR5 expression which allows them to move toward the edge of the follicle at the T:B interface (48). Additionally, both cell types upregulate Epstein-Barr virus-induced G protein coupled receptor 2 (EBI2) which facilitates their localization at the T:B border (49–51). Stromal cells at the inner- and outer-follicle regions regulate the oxysterol ligands for EBI2 which facilitates the co-localization of $EBI2^+$ T and B cells enabling cognate T:B interactions (8, 51–54).

The final step for GC formation requires activated $CD4^+$ T cells and B cells to migrate to the follicle from the T:B border as GC B cells and fully differentiated Tfh cells. Both $CD4^+$ T cells and B cells downregulate CCR7 and EBI2 whilst stably expressing CXCR5. This enables them to escape the chemotactic pull of the T cell zone and outer follicle in order to move into the center of the follicle (14, 51, 55, 56). Both $CD4^+$ T and B cells upregulate S1P

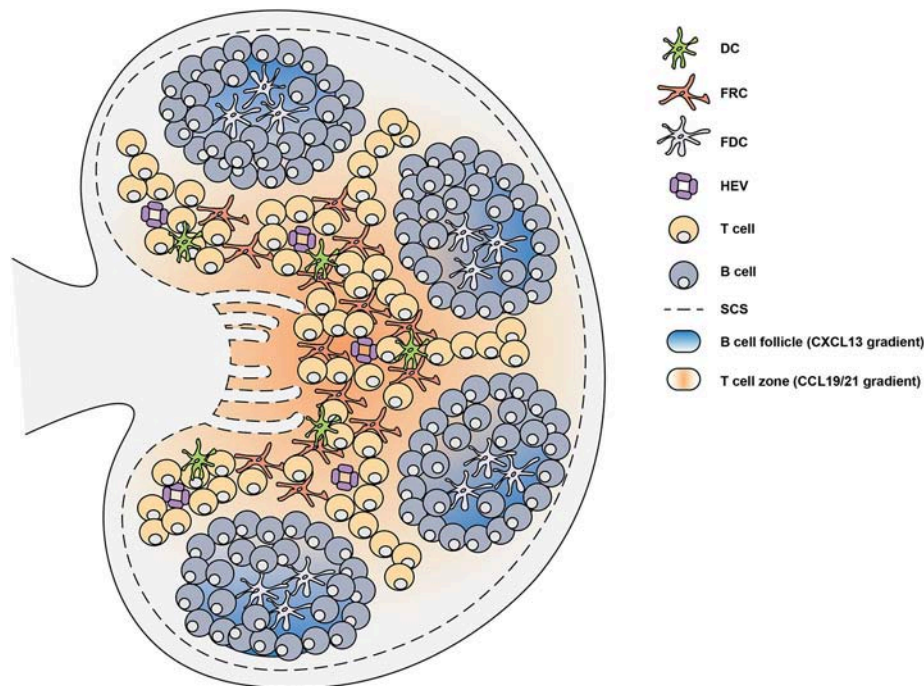


FIGURE 2 | Lymph node structure is supported by stromal cells. Secondary lymphoid organs are divided into distinct regions through the generation of chemokine gradients by stromal cells. In the lymph node (LN), these chemokine gradients allow the circulation of lymphocytes which enter through high endothelial venules (HEV). In the T cell zone, fibroblastic reticular cells (FRCs) generate a CCL19 and CCL21 gradient which facilitates the migration of T cells and dendritic cells (DCs). The B cell follicles contain follicular dendritic cells (FDCs) which generate a CXCL13 gradient that promote the localization of B cells. Upon infection or immunization, antigen can enter the LN through the subcapsular sinus (SCS) or can be brought by DCs to trigger the activation of lymphocytes.

receptor 2 (S1PR2) that supports their localization to the follicle center by binding S1P, which is present in low concentrations within the follicle, and reduces their responsiveness to other chemoattractants (57, 58). Loss of S1PR2 results in B cells losing their ability to accurately localize at the center of the follicle and the combined loss of S1PR2 and CXCR5 abrogates T cell localization to the GC (57, 58). At the center of the follicle, signals exchanged between T and B cells provide the final cues for Tfh cell differentiation and promote B cell proliferation to seed the GC (59). Once the GC response is initiated, both the FDC and CRC networks expand and divide the GC into the two distinct light and dark zones. Both the CRC and FDC networks are essential in maintaining the function and structure of the GC while orchestrating the interactions between different immune cells of the GC.

The Role of Follicular Stromal Cells in the Regulation of the GC Response

The network of CRCs was recently discovered due to their high expression of CXCL12 in the DZ and they were found to have low network density as well as a net-like morphology (19, 37). These cells are distinct from FDCs and FRCs as they do not express the typical FDC/FRC markers which include CD35, ERTR7, FDC-M1/M2, FcγRII, and VCAM1 (37). Due to the lack of antigen-capture mediators such as CD35 and FcγRII on the surface of

CRCs, it is likely that CRCs do not function as antigen presenting cells but meet another specialized requirement in the DZ niche. Thus, CXCL12 production is believed to be one of the essential functions of CRCs in the GC DZ (27). Moreover, two-photon laser-scanning microscopy revealed that GC B cells are able to crawl in and around CRC networks, which depend on CXCR4 signaling for their distribution (37). Therefore, CRCs likely provide support for GC B cells through structural maintenance of the DZ in addition to generating a CXCL12 gradient within the GC. However, the precise role of CRCs in the GC remains to be fully elucidated.

In contrast to the CRCs, FDCs were discovered in the 1960s and are better characterized. During GC formation, the expansion of FDCs is mainly thought to be driven by proliferation of MRCs and their subsequent differentiation into FDCs (60). Throughout this process the FDCs also become activated through TLR4 (61, 62) and B-cell derived lymphotoxin (LT) $\alpha 1\beta 2$ signaling (63), though the precise mechanisms remain unidentified. Once activated, FDCs begin to increase their expression of CXCL13 and BAFF, which support GC development and maintenance of the LZ (27). Studies in mice have shown that ablation of FDCs results in GC termination due to reduced survival and localization of GC B cells; therefore FDCs are absolutely necessary for the GC response (64). The activation of FDCs also triggers an increase in their expression of antigen-capture molecules such as CD35, CD21, and FcγRII

(27). These molecules are critical for the long-term retention and display of antigen on the surface of the FDC network. This allows antigen-specific B cells to test their Ig receptors by capturing antigen from FDCs to subsequently internalize, process and present the antigen peptides to Tfh cells in order to receive critical survival signals (17, 42). FDCs can also produce cytokines such as IL-6 (65, 66) and IL-15 (67, 68) that promote SHM and IgG production and support B cell proliferation, respectively. Additionally, FDC production of FDC-M1 aids in the clearance of apoptotic GC B cells as FDC-M1 coats B cells, marking them for clearance by tangible-body macrophages (69).

Stromal Cells Regulate Tfh and GC B Cell Interactions Within the GC

The CRC and FDC networks form distinct niches critical for the structural support and maintenance of the GC. These two stromal cell subsets are also crucial for the localization of GC B cells within the GC and form a spatially segregated stage where T and B cells can undergo crucial interactions to promote the generation of high-affinity antibody-secreting plasma cells and memory B cells. During the GC response, GC B cells shuttle between the DZ and LZ using a timed program (19). GC B cells can localize in the DZ through their expression of CXCR4 in response to CXCL12 (16). However as they proliferate they downregulate CXCR4 and upregulate CXCR5, which together with the FDC-mediated CXCL13 gradient, enables them to move toward the LZ (16, 19). Migration to the LZ is necessary for centrocytes to acquire antigen and present it to Tfh cells in order for high-affinity B cell clones to survive (3). Through signals received in the LZ, a subset of centrocytes is then able to re-express CXCR4 and migrate back to the DZ via CXCL12-mediated migration where they can undergo further rounds of proliferation and SHM (19). This results in bidirectional B cell trafficking between the two zones that allows for multiple rounds of proliferation and selection to further refine the affinity of responding GC B cells (17, 19).

Tfh cells are conventionally thought to localize to the LZ through their high-expression of CXCR5. For Tfh cells to be retained in the GC they must express not only S1PR2, but also SLAM-associated protein (SAP), which promotes antigen-specific T-B adhesion (70, 71). SAP-deficient T cells are able to localize to the follicle through their expression of CXCR5, but once in the follicle they exhibit severe defects in GC recruitment and retention (71). Additionally, GC retention of Tfh cells can be mediated by expression of a class B Ephrin, EFN1, which negatively controls Tfh cell retention and also promotes interleukin (IL-21) production (72). While these studies have investigated mechanisms by which Tfh cells are retained within the GC, the functional importance of their localization within the GC compartments remains largely unexplored. GC Tfh cells are able to co-express both CXCR4 and CXCR5 (73). The expression of CXCR4 by Tfh cells has been shown to determine their localization between the LZ and DZ (73). Moreover, *in vitro* studies with human immune cells isolated from tonsils have shown FDCs may play a role in modulating CXCR4 expression on T cells (74). Another study also showed that Tfh cells

which express IL-21 have high expression of CXCR4 and are able to localize closer to the DZ (75). However, the functional significance of differential CXCR4 expression of Tfh cells and their localization within the GC remains unknown largely due to the importance of CXCR4 in thymic maturation of T cells (76). Thus, GC stromal cells also play a role in directing the localization of Tfh cells.

Chemokine secretion by the stromal cell networks of SLOs is essential for the regulation of various aspects of the immune system, ranging from the homeostatic migration of lymphocytes to the initiation and maintenance of the GC response. Within the GC reaction, stromal cells provide chemokine cues that promote B cell trafficking between the different GC compartments as well as supplying antigen crucial for affinity maturation. However, whether the different stromal cell subsets of the GC can regulate the function of Tfh cells remains to be explored. Further study into the mechanisms by which stromal cells can regulate the GC will lead to a better understanding of the events required for optimal GC responses against infection and vaccination.

REGULATION OF GC RESPONSES BY T FOLLICULAR REGULATORY CELLS

While the specialized formation of the GC and T–B cell crosstalk are critical to provide protection against a broad range of invading pathogens, the stochastic nature of SHM makes the generation of cross-/self-reactive B cell clones a by-product of GC responses to foreign antigens (77). This can lead to the development of autoimmune disease. The importance of Treg cells for the control of both autoimmune and antibody responses has been long known (78–81). Mice and humans with loss-of-function mutations in the *Foxp3* gene do not form Treg cells and suffer from a fatal early-onset T cell-dependent, lymphoproliferative disorder manifested by autoantibody-mediated autoimmunity (diabetes, thyroiditis, haemolytic anemia) and increased levels of circulating antibodies (82–86). The link between antibody production and Treg cells lead researchers to identify a subset of Treg cells that gain access to the B cell follicle and participate in the regulation of the GC response (87–89). These T follicular regulatory (Tfr) cells simultaneously express markers of Treg and Tfh cells and have suppressive function (87–91). Since their discovery, Tfr cells have been regarded as putative key GC regulators that fine tune the response.

Tfr Cell Differentiation

Tfr cells are derived from *Foxp3*⁺ precursors; the majority come from thymic Treg cells, but they can also arise from naïve T cells when immunization conditions favor induced Treg development (92, 93). The differentiation of Tfr cells is not characterized as well as the differentiation of Tfh cells, but it appears that they also undergo a multistep Bcl-6-dependent differentiation process like Tfh cells. Like other naïve CD4⁺ T cells, antigen presentation by DCs is required for Tfr cell differentiation (88, 92, 94, 95), along with positive co-stimulatory signals through CD28 and ICOS (59, 96–101). However, the DC subsets directly responsible

for stimulating Tfr cell differentiation remain unclear. The differentiation into GC Tfr cells is also dependent on B-cell interactions (88, 94). However, B cells appear to be required only for final stages of Tfr cell differentiation, as putative Tfr cells were found in the blood of μ MT mice following immunization and B-cell deficiency patients (BTK deficiency) (94, 102).

Despite some similarities, there are also differences in the differentiation requirements of Tfr and Tfh cells. The negative co-stimulatory molecules PD-1 and CTLA-4 impact Tfr cell generation. PD-1 signaling selectively inhibits thymic Treg cell differentiation into Tfr cells, prior to B-cell interactions in a PD-L1-dependent manner (100), while blockade of PD-L1 signals in the periphery inhibits the generation of induced Tfr cells (92). Deletion of CTLA-4 leads to an increase in the frequency and absolute numbers of Tfr cells (103, 104). However, it is still unknown whether CTLA-4 impairs Tfr cell differentiation or maintenance, or whether the increased Tfr cell numbers are simply due to an increased GC response overall. IL-21 is a key helper cytokine produced by Tfh cells, which has a negative impact on Tfr cell numbers (105, 106), suggesting that Tfh cells evoke a feedback mechanism to control Tfr cell numbers via this cytokine (107, 108). Mechanistically, Jandl and colleagues propose that IL-21 induces Bcl-6 expression which in turn limits CD25, and the reduction of CD25 expression then leads to lower responsiveness to IL-2, consequently restraining Tfr cell expansion (105). However, because it has been shown that Tfr cells do not express CD25, the high affinity IL-2 receptor (109–111), it is more likely that IL-21 would limit Tfr cell precursors, rather than fully differentiated Tfr cells. CD25 expression limits Tfr differentiation through induction of Blimp-1 (109), a transcription factor known to repress Tfr differentiation (88). Although, IL-2 seems to inhibit Tfr cell differentiation, the absence of IL-2/STAT5 signaling may lead to Foxp3 downregulation (112). Therefore, the maintenance of Foxp3-expressing Treg cells in the absence of IL-2/CD25 (IL-2R α) must be accomplished by other homeostatic mechanisms, such as high amounts of intermediate-affinity IL-2 receptor (CD122/IL-2R β), which may be sufficient to prevent Foxp3 downregulation (109). Whether CD25⁺ and CD25[−] Tfr cells represent two stages of differentiation or two functionally and biologically distinct cell subsets is still unknown.

Tfr cell differentiation culminates in the expression of Bcl-6, the master transcriptional regulator for Tfr cell differentiation (87–89). However, it is not the only transcription factor that contributes to the Tfr cell fate. Expression of transcription factor nuclear factor of activated T cells 2 (NFAT2, also known as NFATc1) is required for CXCR5 upregulation on Treg cells through binding to the *Cxcr5* promoter (113). Tfr cell differentiation also requires an intricate network of many other molecules, such as stromal interaction molecule 1 (STIM1) and STIM2 (114, 115), tumor necrosis factor receptor (TNFR)-associated factor 3 (TRAF3) (116), signal transducer and activator 3 (STAT3) (117), p85 α -osteopontin, and members of the helix-loop-helix family (E and Id proteins). More recently, mTORC1 signaling was also shown to induce *de novo* Tfr cell differentiation from thymic-derived Treg cells (118).

Identity and Specificity of Tfr Cells

The first studies describing Tfr cells reported that these cells are derived from thymic Treg cells (87–89). Recent evidence further supports the preferential origin of Tfr cells from thymic Foxp3⁺ Treg cells (93), but Tfr cells can also be derived from induced Treg cells, that arise from the induction of Foxp3 expression in naive CD4⁺ T cells (92). Tfr cell recruitment into the GC and their suppressive capacity occurs in response to immunization, but Tfr cells do not need to be specific for the immunizing antigen (93, 94). However, a proportion of Tfr cells can also be specific for the immunizing antigen (92), suggesting that Tfr cells can arise through a number of pathways. TCR repertoire analysis at the population level showed that Tfr cells are an oligoclonal population and have a TCR repertoire that is more similar to the repertoire of Treg cells than Tfh cells (93, 119). How Tfr cells are recruited to the GC upon immunization, with TCR specificities that are irrelevant to the immunizing antigen, is a significant unknown in the biology of these cells.

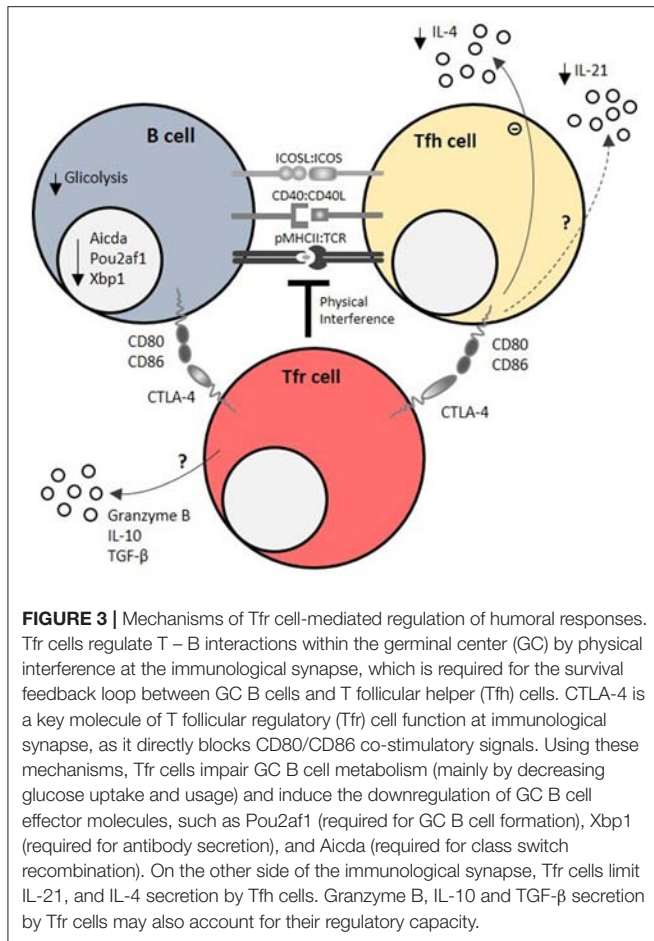
One remaining question regarding the regulation of humoral immunity is the overall contribution of extrafollicular responses to the generation of B cell autoreactivity and its regulation. It is not clear to which extent extrafollicular sites contribute to the emergence of autoreactivity in many diseases, and it remains unknown whether specialized regulatory mechanisms are in place at those locations.

Mechanisms of Tfr Cell Function

Tfr cells specialize in the regulation of the GC response by directly modulating Tfh cell proliferation, B cell metabolism and cytokines secreted by Tfh cells in secondary lymphoid organs. Thus, Tfr cells modify GC outcomes at several levels: (a) control of GC size; (b) selection of antigen specific Tfh and B cell clones; and (c) modulation of class switch and affinity maturation of antibodies (**Figure 3**).

The precise molecules that underpin such effects are largely unknown, but undoubtedly encompass CTLA-4-mediated suppression (103, 104). CTLA-4 has a widely known function in maintaining immune homeostasis and mediating Treg cell function (81, 120–122). Mice that lack CTLA-4 on Treg cells have spontaneous GC responses (104), however in these experiments the caveat is that CTLA4 is lost on all Treg cells, not only Tfr cells. Tamoxifen-induced CTLA-4 depletion on Treg cells at the time of immune challenge led to an expansion of GC B, Tfr, and Tfh cells due to defective Treg cell function (103). Whether CTLA-4 mediated CD80/CD86 transendocytosis plays a role in Tfr cell function within GCs is still controversial (103, 104). Despite the described role of CTLA-4 in mediating Tfr cell function, it is expected that these cells employ multiple and complementary regulatory mechanisms such as TGF- β , IL-10 (88, 94) and granzyme B secretion (88, 123) (**Figure 3**) to perform their suppressive functions.

The suppressive functions of Tfr cells seem to act directly on the metabolic pathways of B and Tfh cells (94, 100, 103, 106). Using several *in vitro* systems, it was shown that Tfr do not affect the transcriptomic signature or activation potential of B cells or Tfh cells, however these cells lose the ability to express key effector molecules, such as Pou2af1, Xbp1 and Aicda, in the presence of



Tfr cells (106). It appears that Tfr cell-imposed B cell modulation persists in the absence of Tfr cells due to epigenetic changes. However, IL-21 was able to overcome the suppressive effect of Tfr cells by both increasing B cell metabolism and inhibiting Tfr cells (106).

While initial studies ascribed the control of GC size, class-switching and antibody affinity to Tfr cells (87–89, 124), more recent studies support the concept that Tfr cells can restrain the generation of antigen-specific antibodies while favoring the emergence of high affinity antibody-secreting B cells (103, 104, 109, 124, 125). Indeed, CTLA-4 competent Tfr cells seem to impose more stringent B cell competition for Tfh cell help (103, 104). Additionally, the outcome of the GC reaction can also be correlated with age-induced alterations in Tfh and Tfr cell numbers and function (126). In aged mice, reduced titers of NP-specific antibodies following NP-OVA immunization were associated with defective Tfh cell function and higher proportions of highly suppressive Tfr cells (126).

These regulatory mechanisms have been further studied in murine models of autoimmune disease, where Tfr cells were directly implicated in ensuring tolerance to self-antigens and preventing autoimmunity (109, 113, 115, 127). Thus, the selective use of Tfr cells (or IL-2 to fine-tune Tfr cell responses) might

be a novel way to therapeutically intervene in diseases where pathogenic GC reactions are the cause of underlying pathology.

Taken together, it appears that cognate interactions are required for Tfr-mediated regulation, while the specific nature of the cellular interactions are yet to be fully characterized. Results from *in vitro* experimental systems devoid of any antigen-presenting cells besides B cells suggest that B-Tfr interactions can trigger regulation that is sufficient to overcome the positive signals delivered by co-cultured Tfh cells (106).

Division of Labor Between Treg and Tfr Cells

Although we have a good understanding of broad Treg cell biology, it is still unclear how different Treg cell subsets integrate to underpin immune tolerance and regulation of humoral responses. It is clear that the absence of Foxp3⁺ Treg cells leads to uncontrolled and spontaneous humoral responses (128–131), however, the contribution of CXCR5⁺ Tfr cells to this overall pathology is not known. Nevertheless, humoral suppressive capacity has been assigned preferentially to Tfr cells. This concept arose from observations where conventional (non-Tfr) Treg cells lacked the capacity to suppress Tfh cell proliferation, B cell activation, and class switch recombination (94, 106). Conversely, two independent groups found a comparable decrease in Tfh cell proliferation when co-cultured with Tfr and conventional Treg cells (87, 104). Hence, while a direct comparison of Tfr and conventional Treg cells in physiological conditions is lacking, Tfr cells presumably acquire their unique humoral suppressive capacity when they co-opt the Tfh cell differentiation program. The suppression of Tfh cell proliferation is probably not unique to Tfr cells, as it might be a general Treg cell feature. However, one critical aspect that might distinguish Tfr from conventional Treg cells *in vivo* is the exceptional ability of Tfr cells to access the GC.

Blood and Human Tfr Cells

GC reactions are orchestrated in secondary lymphoid organs, but in the blood of mice circulating Tfr cells-like cells have also been described (94). This adds an additional layer of complexity, as different immune compartments might evoke different Tfr cell responses (94, 100, 132–135). The population of circulating ICOS^{lo} Tfr cells were shown to behave as memory cells and have less suppressive capacity. They originate after priming by DCs, but without full commitment to the GC fate (94, 100). This suggests that Tfr cell effector activity is initiated during contact with DCs in the T cell zone, strengthened in the inter-follicular region during contact with B cells, and optimized in the GC. However, it is not clear where exactly Tfr cells modulate GC reactions, especially in humans. Recently, human Tfr cells were found to be preferentially distributed at the periphery of GCs (136). While the same has also been shown in murine models (100), it is still unknown whether human Tfr cells share all the biological features of murine Tfr cells. For instance, in human lymph nodes, Tfr cells are not PD-1⁺CD25[−] like in mice (100, 109, 136).

Although a CD69[−] human tonsil Treg cell subset with B cell suppressive function was discovered before the identification of

Bcl-6⁺Foxp3⁺ Tfr cells in mice (137, 138), the restricted access to human secondary lymphoid tissues forced the search for putative Tfr cells in human blood. Several studies have focused on circulating CXCR5⁺Foxp3⁺ T cells to define Tfr cells in humans with different diseases (90). We recently established the biology and ontogeny of human blood CXCR5⁺Foxp3⁺ Tfr cells (102). Human blood CXCR5⁺Foxp3⁺ Tfr cells comprise Tfr cell precursors arising from secondary lymphoid tissues prior to B-cell interactions. Thus, human blood Tfr cells are predominantly CD45RO⁻ naïve cells not yet endowed with full B cell and humoral regulatory functions. Furthermore, autoimmune diseases may be associated with different types of dysregulation of the GC response. Therefore, it is likely that different alterations of Tfr frequency, distribution, and function will be found in different autoimmune diseases.

Peyer's Patches: Specialized Germinal Centers in a Unique Anatomical Location

GCs in Peyer's patches (PPs) are unique due to their special anatomical location and functions. They are influenced by the gut microbiota, and in return produce IgA antibodies which contribute to the control of gut microbial homeostasis. Due to their special environment and function, these GCs require specialized forms of regulation (139).

Peyer's patches are non-encapsulated lymphoid tissues associated with the small intestinal epithelium. In mice, 6–12 PPs are interspersed along the whole length of the small intestine, while the human intestine is associated with 100–200 PPs (139). PPs are continuously exposed to antigenic stimulation by the commensal microbiota. The intimate cross talk with the gut microbiota is what sets PPs apart from other lymphoid tissues. The gut microbiome is a complex mix of bacteria, fungi, viruses and protozoa, which populates the whole intestine. Constant stimulation through this microbiota drives the formation of constitutively active GCs in PPs. These GCs produce antibodies against infectious pathogens, but also generate commensal-specific IgA antibodies that promote homeostasis of the gut microbiome (140).

PPs as Places for TD IgA Production

PPs are an important site for T cell dependent IgA production (139). Like other GCs, B cells within PP GCs undergo somatic hypermutation of the Ig locus, followed by selection of B cells bearing BCRs that bind antigen with high affinity. One key difference to peripheral LNs, however, is that in PPs class-switch recombination (CSR) to the IgA isotype occurs (141). IgA antibodies exist as dimers and are secreted at all mucosal surfaces. In the gut this is mediated by M cells in the sub-epithelial dome of PPs. Once in the gut, IgAs bind to a wide variety of commensal bacteria and alter the composition of the microbiome through a variety of mechanisms (140). These include blocking antigen interactions with the host, trapping antigens in the intestinal mucus or interfering with invasive properties of pathogens (140). In addition, IgA antibodies assist with the controlled intestinal uptake of bacterial antigens to boost antigen-specific gut immune response (142, 143). In AID-deficient animals that lack CSR and SHM, there is aberrant expansion of anaerobic

gut commensals and extensive immune hyperplasia (144, 145). Patients with selective IgA deficiency also exhibit changes in their gut microbiome, associated with increased Th17-cell associated inflammation (146). This demonstrates the key role that switched antibody responses play in gut health.

What is not clear is whether this IgA needs to come from the GC response. Evidence suggesting this is not the case comes from studies in which mice lack either T-dependent immune responses (CD28-deficient mice and CD40-deficient mice) or Tfh cells (*Bcl6*^{flx/flx} *Cd4*^{cre/+}). These animals have high IgA antibody titers, near-to-normal levels of bacterial IgA coating, and relatively normal composition of the microbiota (147–149). However, SHM of IgA antibodies mainly occurs in GCs and analysis of mice that express a variant of AID that can facilitate CSR, but not SHM, revealed that this strain exhibited aberrant expansion of commensal bacteria and increased bacterial translocation into mesenteric LNs (150). This suggests that GC responses in the PP can play an important role in the maintenance of microbial homeostasis.

Immune Regulation of GCs in PPs

Given the distinct architecture and location of PPs, their regulatory mechanisms are unique from those in lymph node GCs. Most importantly, Tfh and Tfr cells in PPs are responsive to modulation by the gut microbiota. The ensuing plasticity in T cell regulation allows PP GCs to respond adequately to intestinal infections or changes in the gut microbiota.

Immune Regulation of PPs by Tfh Cells

PPs provide a unique environment for Tfh cell differentiation, where the “rules” established for Tfh cell development are frequently broken. Exclusively in the gut, Tfh cells can derive from RORγt⁺ Th17 cells (151) and Foxp3⁺ Treg cells (152). The precise mechanism for this is unclear, but it may be driven by stimuli from the microbiota, as microbial sensing plays an important role for Tfh differentiation in the gut. As such, the Th17 cell-promoting segmented filamentous bacteria (SFB) were shown to drive the differentiation of PP Tfh cells. Further, microbial ATP controls Tfh cell differentiation in PPs via interactions with the ATP-gated ionotropic P2X7 receptor (153). The egress of these “unusual” PP Tfh cells into systemic sites can have dire consequences for health, as they were reported to exacerbate the auto-antibody responses in arthritis (154). This demonstrates the ability of intestinal Tfh cells to integrate multiple signals from the gut microbiota for their development, with implications not only for gut, but also systemic immunity. Therefore, control of Tfh cell development, and their maintained residence in the gut is critical for organismal health.

Immune Regulation of PPs by Tfr

Similar to Tfh cells, PP Tfr cells have gut-specific features. In PP GCs there is an increased Tfh/Tfr ratio compared to peripheral GCs (155), making the PP resemble early stages of a GC. This has been proposed to enable the expansion of low affinity B cell clones early in the response (156). This is consistent with the proposal of Reboldi et al. (139) who suggest that GCs in PPs resemble the early stages of a GC in order to favor the quick generation of

diverse low-affinity antibodies in response to microbial antigens. Interestingly, gene expression profiling of Tfr cells from PPs and pLNs revealed the surprising finding that PP Tfr cells express the helper cytokine IL-4, unlike LN Tfr cells (157). This could point to a different, potentially less suppressive, role of Tfr cells within PPs.

As discussed above, Tfr cells are considered to be negative regulators of the GC response, but the data about their functionality in PPs is not clear. STAT3-KO mice, which lack Tfr cells, but have PP Tfh cells, have no observable changes in PP GC size or IgA production in the gut (117). However, in an adoptive transfer model Kawamoto et al. implicated Tfr cells in the regulation of IgA-mediated control of the gut microbiome: Supplying T cell-deficient hosts with Treg cells increased IgA production and induced dramatic changes in the composition of the microbiota (125). This is consistent with the observation that depletion of Treg cells results in a drop in IgA levels (158). Together, this suggests that both Tfr functionality as well as the Tfh/Tfr ratio in PPs are adjusted to allow for optimal control of the gut microbiota, although further work is required to precisely define the role for Tfr cells in PPs.

Regulation of PPs by the Microbiota

The gut microbiota is a crucial, but often underappreciated, regulator of the GC response in the gut and the systemic immune system. Germ-free mice, which lack any form of bacterial colonization, exhibit evident deficits in the maturation of their gut associated lymphoid tissues, including PPs and mesenteric lymph nodes. Their PPs are small and produce limited amounts of IgA antibodies (159). In addition, these mice are more susceptible to enteric infections and their systemic immune response to infections is also stunted (160, 161). This demonstrates a strong dependency of the immune system on the microbiota. There is evidence that some bacteria and their products directly affect the GC response in PPs. Transfer of a diverse microbiota into wild-type mice increases GC B cell numbers as well as bacterial IgA-coating (125). Bacterial products can also directly act on immune cells in the PP. Microbial ATP controls Tfh cell differentiation (153) and short-chain fatty acids, a diverse group of bacterial metabolites, were shown to boost plasma cell differentiation and intestinal antibody production

in PPs (162, 163). This demonstrates the strong impact of the microbiota on the GC response. Thus, the interplay of the immune system with the microbiota cannot be neglected when studying the regulation of intestinal GCs.

CONCLUSION

The importance of the GC response for humoral immunity has been known for several decades. However, the cellular and molecular mechanisms that regulate GC function are still being elucidated. This review highlights several known mechanisms by which GCs are regulated through the collaboration of multiple cell types in both LNs and PPs. Given the participation of GCs in physiological and pathological immune responses, a better understanding of GC regulation is likely to have clinical applications. In this respect, it is fundamental to consider and further characterize the complex cellular network and interplay that ultimately control the outcome of GC responses in specific anatomic locations. Further elucidation of the mechanisms which govern GC regulation will be beneficial to improve patient stratification in immune-mediated diseases, and for the identification of novel therapeutic biomarkers.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

ACKNOWLEDGMENTS

MS, SK, and AS-C are funded by the European Union's Horizon 2020 research and innovation programme ENLIGHTEN under the Marie Skłodowska-Curie grant agreement No.: 675395. LG is funded by FAPESP/19906/2014, PTDC/IMI-IMU/7038/2014, and LISBOA-01-0145-FEDER-007391, projeto cofinanciado pelo FEDER através POR Lisboa 2020–Programa Operacional Regional de Lisboa, do PORTUGAL 2020, e pela Fundação para a Ciência e a Tecnologia. ML is supported by the Biotechnology and Biological Sciences Research Council (BBS/E/B/000C0407, BBS/E/B/000C0427).

REFERENCES

- MacLennan ICM. Germinal centers. *Annu Rev Immunol.* (1994) 12:117–39. doi: 10.1146/annurev.iy.12.040194.001001
- Pitzalis C, Jones GW, Bombardieri M, Jones SA. Ectopic lymphoid-like structures in infection, cancer and autoimmunity. *Nat Rev Immunol.* (2014) 14:447–62. doi: 10.1038/nri3700
- Mesin L, Ersching J, Vitoria GD. Germinal center B cell dynamics. *Immunity* (2016) 45:471–82. doi: 10.1016/j.immuni.2016.09.001
- Allen CDC, Cyster JG. Follicular dendritic cell networks of primary follicles and germinal centers: phenotype and function. *Semin Immunol.* (2008) 20:14–25. doi: 10.1016/j.smim.2007.12.001
- Batista FD, Harwood NE. The who, how and where of antigen presentation to B cells. *Nat Rev Immunol.* (2009) 9:15–27. doi: 10.1038/nri2454
- Carrasco YR, Batista FD. B cells acquire particulate antigen in a macrophage-rich area at the boundary between the follicle and the subcapsular sinus of the lymph node. *Immunity* (2007) 27:160–71. doi: 10.1016/j.immuni.2007.06.007
- Phan TG, Grigorova I, Okada T, Cyster JG. Subcapsular encounter and complement-dependent transport of immune complexes by lymph node B cells. *Nat Immunol.* (2007) 8:992–1000. doi: 10.1038/nri1494
- Okada T, Miller MJ, Parker I, Krummel MF, Neighbors M, Hartley SB, et al. Antigen-engaged B cells undergo chemotaxis toward the T zone and form motile conjugates with helper T cells. *PLoS Biol.* (2005) 3:e150. doi: 10.1371/journal.pbio.0030150
- Garside P, Ingulli E, Merica RR, Johnson JG, Noelle RJ, Jenkins MK. Visualization of specific B and T lymphocyte interactions in the lymph node. *Science* (1998) 281:96–9.
- Chan TD, Gatto D, Wood K, Camidge T, Basten A, Brink R. Antigen affinity controls rapid T-dependent antibody production by driving the expansion rather than the differentiation or extrafollicular

- migration of early plasmablasts. *J Immunol.* (2009) 183:3139–49. doi: 10.4049/jimmunol.0901690
11. Coffey F, Alabyev B, Manser T. Initial clonal expansion of germinal center B cells takes place at the perimeter of follicles. *Immunity* (2009) 30:599–609. doi: 10.1016/j.immuni.2009.01.011
 12. Takemori T, Kaji T, Takahashi Y, Shimoda M, Rajewsky K. Generation of memory B cells inside and outside germinal centers. *Eur J Immunol.* (2014) 44:1258–64. doi: 10.1002/eji.201343716
 13. MacLennan ICM, Toellner KM, Cunningham AF, Serre K, Sze DMY, Zúñiga E, et al. Extrafollicular antibody responses. *Immunol. Rev.* (2003) 194:8–18. doi: 10.1034/j.1600-065X.2003.00058.x
 14. Pereira JP, Kelly LM, Xu Y, Cyster JG. EB12 mediates B cell segregation between the outer and centre follicle. *Nature* (2009) 460:1122–6. doi: 10.1038/nature08226
 15. Allen CDC, Okada T, Cyster JG. Germinal-center organization and cellular dynamics. *Immunity* (2007) 27:190–202. doi: 10.1016/j.immuni.2007.07.009
 16. Allen CDC, Ansel KM, Low C, Lesley R, Tamamura H, Fujii N, et al. Germinal center dark and light zone organization is mediated by CXCR4 and CXCR5. *Nat Immunol.* (2004) 5:943–52. doi: 10.1038/nri1100
 17. Vitoria GD, Schwickert TA, Fooksman DR, Kamphorst AO, Meyer-Hermann M, Dustin ML, et al. Germinal center dynamics revealed by multiphoton microscopy with a photoactivatable fluorescent reporter. *Cell* (2010) 143:592–605. doi: 10.1016/j.cell.2010.10.032
 18. Vitoria GD, Dominguez-Sola D, Holmes AB, Deroubaix S, Dalla-Favera R, Nussenzweig MC. Identification of human germinal center light and dark zone cells and their relationship to human B-cell lymphomas. *Blood* (2012) 120:2240–8. doi: 10.1182/blood-2012-03-415380
 19. Bannard O, Horton RM, Allen CD, An J, Nagasawa T, Cyster JG. Germinal center centroblasts transition to a centrocyte phenotype according to a timed program and depend on the dark zone for effective selection. *Immunity* (2013) 39:912–24. doi: 10.1016/j.immuni.2013.08.038
 20. Webb LMC, Linterman MA. Signals that drive T follicular helper cell formation. *Immunology* (2017) 152:185–94. doi: 10.1111/imm.12778
 21. Ballesteros-Tato A, Randall TD. Priming of T follicular helper cells by dendritic cells. *Immunol Cell Biol.* (2014) 92:22–7. doi: 10.1038/icb.2013.62
 22. Chakarov S, Fazilleau N. Monocyte-derived dendritic cells promote T follicular helper cell differentiation. *EMBO Mol. Med.* (2014) 6:590–603. doi: 10.1002/emmm.201403841
 23. Shin C, Han J-A, Koh H, Choi B, Cho Y, Jeong H, et al. CD8 α -dendritic cells induce antigen-specific T follicular helper cells generating efficient humoral immune responses. *Cell Rep.* (2015) 11:1929–40. doi: 10.1016/j.celrep.2015.05.042
 24. Ito T, Wang Y-H, Duramad O, Hori T, Delespesse GJ, Watanabe N, et al. TSLP-activated dendritic cells induce an inflammatory T helper type 2 cell response through OX40 ligand. *J Exp Med.* (2005) 202:1213–23. doi: 10.1084/jem.20051135
 25. Krishnaswamy JK, Gowthaman U, Zhang B, Mattsson J, Szeponik L, Liu D, et al. Migratory CD11b $^{+}$ conventional dendritic cells induce T follicular helper cell-dependent antibody responses. *Sci Immunol.* (2017) 2:eam9169. doi: 10.1126/sciimmunol.aam9169
 26. Kumamoto Y, Hirai T, Wong PW, Kaplan DH, Iwasaki A. CD301b $^{+}$ dendritic cells suppress T follicular helper cells and antibody responses to protein antigens. *Elife* (2016) 5:1–24. doi: 10.7554/eLife.17979
 27. Denton AE, Linterman MA. Stromal networking: cellular connections in the germinal centre. *Curr Opin Immunol.* (2017) 45:103–11. doi: 10.1016/j.coi.2017.03.001
 28. Kufareva I, Salanga CL, Handel TM. Chemokine and chemokine receptor structure and interactions: implications for therapeutic strategies. *Immunol Cell Biol.* (2015) 93:372–83. doi: 10.1038/icb.2015.15
 29. Schulz O, Hammerschmidt SI, Moschovakis GL, Forster R. Chemokines and chemokine receptors in lymphoid tissue dynamics. *Annu Rev Immunol.* (2016) 34:203–42. doi: 10.1146/annurev-immunol-041015-055649
 30. Cremasco V, Woodruff MC, Onder L, Cupovic J, Nieves-Bonilla JM, Schildberg FA, et al. B cell homeostasis and follicle confines are governed by fibroblastic reticular cells. *Nat Immunol.* (2014) 15:973–81. doi: 10.1038/ni.2965
 31. Denton AE, Roberts EW, Linterman MA, Fearon DT. Fibroblastic reticular cells of the lymph node are required for retention of resting but not activated CD8 $^{+}$ T cells. *Proc Natl Acad Sci USA.* (2014) 111:12139–44. doi: 10.1073/pnas.1412910111
 32. Link A, Vogt TK, Favre S, Britschgi MR, Acha-Orbea H, Hinz B, et al. Fibroblastic reticular cells in lymph nodes regulate the homeostasis of naive T cells. *Nat Immunol.* (2007) 8:1255–65. doi: 10.1038/ni1513
 33. Forster R, Schubel A, Breitfeld D, Kremmer E, Renner-Müller I, Wolf E, et al. CCR7 coordinates the primary immune response by establishing functional microenvironments in secondary lymphoid organs. *Cell* (1999) 99:23–33.
 34. Nakano H, Mori S, Yonekawa H, Nariuchi H, Matsuzawa A, Kakiuchi T. A novel mutant gene involved in T-lymphocyte-specific homing into peripheral lymphoid organs on mouse chromosome 4. *Blood* (1998) 91:2886–95.
 35. Yanagihara S, Komura E, Nagafune J, Watarai H, Yamaguchi Y. EB1/CCR7 is a new member of dendritic cell chemokine receptor that is up-regulated upon maturation. *J Immunol.* (1998) 161:3096–102.
 36. Miller 3rd JJ, Nossal GJ. Antigens in immunity. VI. The phagocytic reticulum of lymph node follicles. *J Exp Med.* (1964) 120:1075–1086.
 37. Rodda LB, Bannard O, Ludewig B, Nagasawa T, Cyster JG. Phenotypic and morphological properties of germinal center dark zone Cxcl12-expressing reticular cells. *J Immunol.* (2015) 195:4781–91. doi: 10.4049/jimmunol.1501191
 38. Bajenoff M, Egen JG, Koo LY, Laugier JP, Brau F, Glaichenhaus N, et al. Stromal cell networks regulate lymphocyte entry, migration, and territoriality in lymph nodes. *Immunity* (2006) 25:989–1001. doi: 10.1016/j.immuni.2006.10.011
 39. Pape KA, Catron DM, Itano AA, Jenkins MK. The humoral immune response is initiated in lymph nodes by B cells that acquire soluble antigen directly in the follicles. *Immunity* (2007) 26:491–502. doi: 10.1016/j.immuni.2007.02.011
 40. Roozendaal R, Mempel TR, Pitcher LA, Gonzalez SF, Verschoor A, Mebius RE, et al. Conduits mediate transport of low-molecular-weight antigen to lymph node follicles. *Immunity* (2009) 30:264–76. doi: 10.1016/j.immuni.2008.12.014
 41. Yoshida K, van den Berg TK, Dijkstra CD. Two functionally different follicular dendritic cells in secondary lymphoid follicles of mouse spleen, as revealed by CR1/2 and Fc γ II-mediated immune-complex trapping. *Immunology* (1993) 80:34–39.
 42. Suzuki K, Grigoroa I, Phan TG, Kelly LM, Cyster JG. Visualizing B cell capture of cognate antigen from follicular dendritic cells. *J Exp Med.* (2009) 206:1485–93. doi: 10.1084/jem.20090209
 43. Katakai T, Suto H, Sugai M, Gonda H, Togawa A, Suematsu S, et al. Organizer-like reticular stromal cell layer common to adult secondary lymphoid organs. *J Immunol.* (2008) 181:6189–200. doi: 10.4049/jimmunol.181.9.6189
 44. Miyasaka M, Tanaka T. Lymphocyte trafficking across high endothelial venules: dogmas and enigmas. *Nat Rev Immunol.* (2004) 4:360–70. doi: 10.1038/nri1354
 45. Ansel KM, McHeyzer-Williams LJ, Ngo VN, McHeyzer-Williams MG, and Cyster JG. *In vivo*-activated CD4 T cells upregulate CXC chemokine receptor 5 and reprogram their response to lymphoid chemokines. *J Exp Med.* (1999) 190:1123–34.
 46. Griffith JW, Sokol CL, Luster AD. Chemokines and chemokine receptors: positioning cells for host defense and immunity. *Annu Rev Immunol.* (2014) 32:659–702. doi: 10.1146/annurev-immunol-032713-120145
 47. Leon B, Ballesteros-Tato A, Browning JL, Dunn R, Randall TD, Lund FE. Regulation of T(H)2 development by CXCR5 $^{+}$ dendritic cells and lymphotoxin-expressing B cells. *Nat Immunol.* (2012) 13:681–90. doi: 10.1038/ni.2309
 48. Reif K, Ekland EH, Ohl L, Nakano H, Lipp M, Forster R, et al. Balanced responsiveness to chemoattractants from adjacent zones determines B-cell position. *Nature* (2002) 416:94–9. doi: 10.1038/416094a
 49. Gatto D, Brink R. B cell localization: regulation by EB12 and its oxysterol ligand. *Trends Immunol.* (2013) 34:336–41. doi: 10.1016/j.it.2013.01.007
 50. Kelly LM, Pereira JP, Yi T, Xu Y, Cyster JG. EB12 guides serial movements of activated B cells and ligand activity is detectable in lymphoid and nonlymphoid tissues. *J Immunol.* (2011) 187:3026–32. doi: 10.4049/jimmunol.1101262

51. Suan D, Nguyen A, Moran I, Bourne K, Hermes JR, Arshi M, et al. T follicular helper cells have distinct modes of migration and molecular signatures in naive and memory immune responses. *Immunity* (2015) 42:704–18. doi: 10.1016/j.immuni.2015.03.002
52. Hannedouche S, Zhang J, Yi T, Shen W, Nguyen D, Pereira JP, et al. Oxysterols direct immune cell migration via EBI2. *Nature* (2011) 475:524–7. doi: 10.1038/nature10280
53. Liu C, Yang XV, Wu J, Kuei C, Mani NS, Zhang L, et al. Oxysterols direct B-cell migration through EBI2. *Nature* (2011) 475:519–23. doi: 10.1038/nature10226
54. Yi T, Wang X, Kelly LM, An J, Xu Y, Sailer AW, et al. Oxysterol gradient generation by lymphoid stromal cells guides activated B cell movement during humoral responses. *Immunity* (2012) 37:535–48. doi: 10.1016/j.immuni.2012.06.015
55. Gatto D, Paus D, Basten A, Mackay CR, Brink R. Guidance of B cells by the orphan G protein-coupled receptor EBI2 shapes humoral immune responses. *Immunity* (2009) 31:259–69. doi: 10.1016/j.immuni.2009.06.016
56. Haynes NM, Allen CD, Lesley R, Ansel KM, Killeen N, Cyster JG. Role of CXCR5 and CCR7 in follicular Th cell positioning and appearance of a programmed cell death gene-1-high germinal center-associated subpopulation. *J Immunol.* (2007) 179:5099–108. doi: 10.4049/jimmunol.179.8.5099
57. Green JA, Suzuki K, Cho B, Willison LD, Palmer D, Allen CD, et al. The sphingosine 1-phosphate receptor S1P(2) maintains the homeostasis of germinal center B cells and promotes niche confinement. *Nat Immunol.* (2011) 12:672–80. doi: 10.1038/ni.2047
58. Moriyama S, Takahashi N, Green JA, Hori S, Kubo M, Cyster JG, et al. Sphingosine-1-phosphate receptor 2 is critical for follicular helper T cell retention in germinal centers. *J Exp Med.* (2014) 211:1297–305. doi: 10.1084/jem.20131666
59. Vinuesa CG, Linterman MA, Yu D, MacLennan ICM. Follicular Helper T Cells. *Annu. Rev. Immunol.* (2016) 34:annurev-immunol-041015-055605. doi: 10.1146/annurev-immunol-041015-055605
60. Jarjour M, Jorquera A, Mondor I, Wienert S, Narang P, Coles MC, et al. Fate mapping reveals origin and dynamics of lymph node follicular dendritic cells. *J Exp Med.* (2014) 211:1109–22. doi: 10.1084/jem.20132409
61. Garin A, Meyer-Hermann M, Contie M, Figge MT, Buatois V, Gunzer M, et al. Toll-like receptor 4 signaling by follicular dendritic cells is pivotal for germinal center onset and affinity maturation. *Immunity* (2010) 33:84–95. doi: 10.1016/j.immuni.2010.07.005
62. Victoratos P, Lagnel J, Tzima S, Alimzhanov MB, Rajewsky K, Pasparakis M, et al. FDC-specific functions of p55TNFR and IKK2 in the development of FDC networks and of antibody responses. *Immunity* (2006) 24:65–77. doi: 10.1016/j.immuni.2005.11.013
63. Myers RC, King RG, Carter RH, Justement LB. Lymphotoxin alpha1beta2 expression on B cells is required for follicular dendritic cell activation during the germinal center response. *Eur J Immunol.* (2013) 43:348–59. doi: 10.1002/eji.201242471
64. Wang X, Cho B, Suzuki K, Xu Y, Green JA, An J, et al. Follicular dendritic cells help establish follicle identity and promote B cell retention in germinal centers. *J Exp Med.* (2011) 208:2497–510. doi: 10.1084/jem.20111449
65. Kopf M, Herren S, Wiles MV, Pepys MB, Kosco-Vilbois MH. Interleukin 6 influences germinal center development and antibody production via a contribution of C3 complement component. *J Exp Med.* (1998) 188:1895–906.
66. Wu Y, El Shikh ME, El Sayed RM, Best AM, Szakal AK, Tew JG. IL-6 produced by immune complex-activated follicular dendritic cells promotes germinal center reactions, IgG responses and somatic hypermutation. *Int Immunol.* (2009) 21:745–56. doi: 10.1093/intimm/dxp041
67. Dubois S, Mariner J, Waldmann TA, Tagaya Y. IL-15Ralpha recycles and presents IL-15 in trans to neighboring cells. *Immunity* (2002) 17:537–47. doi: 10.1016/S1074-7613(02)00429-6
68. Park CS, Yoon SO, Armitage RJ, Choi YS. Follicular dendritic cells produce IL-15 that enhances germinal center B cell proliferation in membrane-bound form. *J Immunol.* (2004) 173:6676–83. doi: 10.4049/jimmunol.173.11.6676
69. Kranich J, Krautler NJ, Heinen E, Polymenidou M, Bridel C, Schildknecht A, et al. Follicular dendritic cells control engulfment of apoptotic bodies by secreting Mfge8. *J Exp Med.* (2008) 205:1293–302. doi: 10.1084/jem.20071019
70. Kamperschroer C, Roberts DM, Zhang Y, Weng NP, Swain SL. SAP enables T cells to help B cells by a mechanism distinct from Th cell programming or CD40 ligand regulation. *J Immunol.* (2008) 181:3994–4003. doi: 10.4049/jimmunol.181.6.3994
71. Qi H, Cannons JL, Klauschen F, Schwartzberg PL, Germain RN. SAP-controlled T-B cell interactions underlie germinal centre formation. *Nature* (2008) 455:764–9. doi: 10.1038/nature07345
72. Lu P, Shih C, Qi H. Ephrin B1-mediated repulsion and signaling control germinal center T cell territoriality and function. *Science* (2017) 80:356. doi: 10.1126/science.aai9264
73. Elsnier RA, Ernst DN, Baumgarth N. Single and coexpression of CXCR4 and CXCR5 identifies CD4 T helper cells in distinct lymph node niches during influenza virus infection. *J Virol.* (2012) 86:7146–57. doi: 10.1128/JVI.06904-11
74. Estes JD, Keele BF, Tenner-Racz K, Racz P, Redd MA, Thacker TC, et al. Follicular dendritic cell-mediated up-regulation of CXCR4 expression on CD4 T cells and HIV pathogenesis. *J Immunol.* (2002) 169:2313–22. doi: 10.4049/jimmunol.169.5.2313
75. Weinstein JS, Herman EI, Lainez B, Licona-Limón P, Esplugues E, Flavell R, et al. TFH cells progressively differentiate to regulate the germinal center response. *Nat Immunol.* (2016) 17:1197–205. doi: 10.1038/ni.3554
76. Janas ML, Turner M. Stromal cell-derived factor 1alpha and CXCR4: newly defined requirements for efficient thymic beta-selection. *Trends Immunol.* (2010) 31:370–6. doi: 10.1016/j.it.2010.07.002
77. Ray SK, Putterman C, Diamond B. Pathogenic autoantibodies are routinely generated during the response to foreign antigen: a paradigm for autoimmune disease. *Proc Natl Acad Sci USA.* (1996) 93:2019–24. doi: 10.1073/pnas.93.5.2019
78. Dhaeze T, Stinissen P, Liston A, Hellings N. Humoral autoimmunity: a failure of regulatory T cells? *Autoimmun Rev.* (2015) 14:735–41. doi: 10.1016/j.autrev.2015.04.006
79. Fujio K, Okamura T, Sumitomo S, Yamamoto K. Regulatory T cell-mediated control of autoantibody-induced inflammation. *Front Immunol.* (2012) 3:28. doi: 10.3389/fimmu.2012.00028
80. Ochs HD, Ziegler SF, Torgerson TR. FOXP3 acts as a rheostat of the immune response. *Immunol Rev.* (2005) 203:156–64. doi: 10.1111/j.0105-2896.2005.00231.x
81. Wing JB, Sakaguchi S. Foxp3+ Treg cells in humoral immunity. *Int Immunol.* (2014) 26:61–9. doi: 10.1093/intimm/dxt060
82. Brunkow ME, Jeffery EW, Hjerrild KA, Paepers B, Clark LB, Yasayko S-A, et al. Disruption of a new forkhead/winged-helix protein, scurfy, results in the fatal lymphoproliferative disorder of the scurfy mouse. *Nat Genet.* (2001) 27:68–73. doi: 10.1038/83784
83. Chatila TA, Blaeser F, Ho N, Lederman HM, Voulgaropoulos C, Helms C, et al. JM2, encoding a fork head-related protein, is mutated in X-linked autoimmunity-allergic dysregulation syndrome. *J Clin Invest.* (2000) 106:R75–81. doi: 10.1172/JCI11679
84. Gambineri E, Torgerson TR, Ochs HD. Immune dysregulation, polyendocrinopathy, enteropathy, and X-linked inheritance (IPEX), a syndrome of systemic autoimmunity caused by mutations of FOXP3, a critical regulator of T-cell homeostasis. *Curr Opin Rheumatol.* (2003) 15:430–5. doi: 10.1097/00002281-200307000-00010
85. Ochs HD, Bennett CL, Christie J, Ramsdell F, Brunkow ME, Ferguson PJ, et al. The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. *Nat Genet.* (2001) 27:20–1. doi: 10.1038/83713
86. Wildin RS, Ramsdell F, Peake J, Faravelli F, Casanova JL, Buist N, et al. X-linked neonatal diabetes mellitus, enteropathy and endocrinopathy syndrome is the human equivalent of mouse scurfy. *Nat Genet.* (2001) 27:18–20. doi: 10.1038/83707
87. Chung Y, Tanaka S, Chu F, Nurieva RI, Martinez GJ, Rawal S, et al. Follicular regulatory T cells expressing Foxp3 and Bcl-6 suppress germinal center reactions. *Nat Med.* (2011) 17:983–8. doi: 10.1038/nm.2426
88. Linterman MA, Pierson W, Lee SK, Kallies A, Kawamoto S, Rayner TF, et al. Foxp3+ follicular regulatory T cells control the germinal center response. *Nat Med.* (2011) 17:975–82. doi: 10.1038/nm.2425

89. Wollenberg I, Agua-Doce A, Hernández A, Almeida C, Oliveira VG, Faro J, et al. Regulation of the germinal center reaction by Foxp3+ follicular regulatory T cells. *J Immunol.* (2011) 187:4553–60. doi: 10.4049/jimmunol.1101328
90. Maceiras AR, Fonseca VR, Agua-Doce A, Graca L. T follicular regulatory cells in mice and men. *Immunology* (2017) 152:25–35. doi: 10.1111/imm.12774
91. Sage PT, Sharpe AH. T follicular regulatory cells in the regulation of B cell responses. *Trends Immunol.* (2015) 36:410–8. doi: 10.1016/j.it.2015.05.005
92. Aloulou M, Carr EJ, Gador M, Bignon A, Liblau RS, Fazilleau N, et al. Follicular regulatory T cells can be specific for the immunizing antigen and derive from naive T cells. *Nat Commun.* (2016) 7:10579. doi: 10.1038/ncomms10579
93. Maceiras AR, Almeida SCP, Mariotti-Ferrandiz E, Chaara W, Jebbawi F, Six A, et al. T follicular helper and T follicular regulatory cells have different TCR specificity. *Nat Commun.* (2017) 8:15067. doi: 10.1038/ncomms15067
94. Sage PT, Alvarez D, Godec J, Von Andrian UH, Sharpe AH. Circulating T follicular regulatory and helper cells have memory-like properties. *J Clin Invest.* (2014) 124:5191–204. doi: 10.1172/JCI76861
95. Ting JPY, Trowsdale J. Genetic control of MHC class II expression. *Cell* (2002) 109:S21–33. doi: 10.1016/S0092-8674(02)00696-7
96. Baumjohann D, Preite S, Reboldi A, Ronchi F, Ansel KM, Lanzavecchia A, et al. Persistent antigen and germinal center B cells sustain T follicular helper cell responses and phenotype. *Immunity* (2013) 38:596–605. doi: 10.1016/j.immuni.2012.11.020
97. Choi YS, Kageyama R, Eto D, Escobar TC, Johnston RJ, Monticelli L, et al. ICOS receptor instructs T follicular helper cell versus effector cell differentiation via induction of the Transcriptional Repressor Bcl6. *Immunity* (2011) 34:932–46. doi: 10.1016/j.immuni.2011.03.023
98. Crotty S. Follicular helper CD4 T cells (TFH). *Annu Rev Immunol.* (2011) 29:621–63. doi: 10.1146/annurev-immunol-031210-101400
99. Deenick EK, Chan A, Ma CS, Gatto D, Schwartzberg PL, Brink R, et al. Follicular helper T cell differentiation requires continuous antigen presentation that is independent of unique B cell signaling. *Immunity* (2010) 33:241–53. doi: 10.1016/j.immuni.2010.07.015
100. Sage PT, Francisco LM, Carman CV, Sharpe AH. The receptor PD-1 controls follicular regulatory T cells in the lymph nodes and blood. *Nat Immunol.* (2013) 14:152–61. doi: 10.1038/ni.2496
101. Stone EL, Pepper M, Katayama CD, Kerdiles YM, Lai CY, Emslie E, et al. ICOS coreceptor signaling inactivates the transcription factor FOXO1 to promote Tfh cell differentiation. *Immunity* (2015) 42:239–51. doi: 10.1016/j.immuni.2015.01.017
102. Fonseca VR, Agua-Doce A, Maceiras AR, Pierson W, Ribeiro F, Romão VC, et al. Human blood Tfr cells are indicators of ongoing humoral activity not fully licensed with suppressive function. *Sci Immunol.* (2017) 2:eaan1487. doi: 10.1126/sciimmunol.aan1487
103. Sage PT, Paterson AM, Lovitch SB, Sharpe AH. The coinhibitory receptor CTLA-4 controls B cell responses by modulating T follicular helper, T follicular regulatory, and T regulatory cells. *Immunity* (2014) 41:1026–39. doi: 10.1016/j.immuni.2014.12.005
104. Wing JB, Ise W, Kurosaki T, Sakaguchi S. Regulatory T cells control antigen-specific expansion of Tfh cell number and humoral immune responses via the coreceptor CTLA-4. *Immunity* (2014) 41:1013–25. doi: 10.1016/j.immuni.2014.12.006
105. Jandl C, Liu SM, Cañete PF, Warren J, Hughes WE, Vogelzang A, et al. IL-21 restricts T follicular regulatory T cell proliferation through Bcl-6 mediated inhibition of responsiveness to IL-2. *Nat Commun.* (2017) 8:14647. doi: 10.1038/ncomms14647
106. Sage PT, Ron-Harel N, Juneja VR, Sen DR, Maleri S, Sunnak W, et al. Suppression by TFR cells leads to durable and selective inhibition of B cell effector function. *Nat Immunol.* (2016) 17:1436–46. doi: 10.1038/ni.3578
107. Ettinger R, Kuchen S, Lipsky PE. The role of IL-21 in regulating B-cell function in health and disease. *Immunol Rev.* (2008) 223:60–86. doi: 10.1111/j.1600-065X.2008.00631.x
108. Lühje K, Kallies A, Shimohakamada Y, Belz GT, Light A, Tarlinton DM, et al. The development and fate of follicular helper T cells defined by an IL-21 reporter mouse. *Nat Immunol.* (2012) 13:491–8. doi: 10.1038/ni.2261
109. Botta D, Fuller MJ, Marquez-Lago TT, Bachus H, Bradley JE, Weinmann AS, et al. Dynamic regulation of T follicular regulatory cell responses by interleukin 2 during influenza infection. *Nat Immunol.* (2017) 18:1249–60. doi: 10.1038/ni.3837
110. Ritvo PGG, Churlaud G, Quiniou V, Florez L, Brimaud F, Fourcade G, et al. Tfr cells lack IL-2R α but express decoy IL-1R2 and IL-1Ra and suppress the IL-1-dependent activation of Tfh cells. *Sci. Immunol.* (2017) 2:eaan0368. doi: 10.1126/sciimmunol.aan0368
111. Wing JB, Kitagawa Y, Locci M, Hume H, Tay C, Morita T, et al. A distinct subpopulation of CD25(-) T-follicular regulatory cells localizes in the germinal centers. *Proc Natl Acad Sci USA.* (2017) 114:E6400–9. doi: 10.1073/pnas.1705551114
112. Josefowicz SZ, Lu LF, Rudensky AY. Regulatory T cells: mechanisms of differentiation and function. *Annu Rev Immunol.* (2012) 30:531–64. doi: 10.1146/annurev.immunol.25.022106.141623
113. Vaeth M, Müller G, Stauss D, Dietz L, Klein-Hessling S, Serfling E, et al. Follicular regulatory T cells control humoral autoimmunity via NFAT2-regulated CXCR5 expression. *J Exp Med.* (2014) 211:545–61. doi: 10.1084/jem.20130604
114. Feske S, Skolnik EY, Prakriya M. Ion channels and transporters in lymphocyte function and immunity. *Nat Rev Immunol.* (2012) 12:532–47. doi: 10.1038/nri3233
115. Vaeth M, Eckstein M, Shaw PJ, Kozhaya L, Yang J, Berberich-Siebelt F, et al. Store-operated Ca²⁺ entry in follicular T cells controls humoral immune responses and autoimmunity. *Immunity* (2016) 44:1350–64. doi: 10.1016/j.immuni.2016.04.013
116. Chang J-H, Hu H, Jin J, Puebla-Osorio N, Xiao Y, Gilbert BE, et al. TRAF3 regulates the effector function of regulatory T cells and humoral immune responses. *J Exp Med.* (2014) 211:137–51. doi: 10.1084/jem.20131019
117. Wu H, Xie MM, Liu H, Dent AL. Stat3 is important for follicular regulatory T cell differentiation. *PLoS ONE* (2016) 11:e0155040. doi: 10.1371/journal.pone.0155040
118. Xu L, Huang Q, Wang H, Hao Y, Bai Q, Hu J, et al. The Kinase mTORC1 promotes the generation and suppressive function of follicular regulatory T cells. *Immunity* (2017) 47:538–551.e5. doi: 10.1016/j.immuni.2017.08.011
119. Ritvo PGG, Chaara W, Soufi K, El Bonnet B, Six A, Mariotti-Ferrandiz E, et al. High-resolution repertoire analysis of Tfr and Tfh cells reveals unexpectedly high diversities indicating a bystander activation of follicular T cells. *bioRxiv* (2017) 231977. doi: 10.1101/231977
120. Kajsia W, Onishi Y, Prieto-Martin P. CTLA-4 control over Foxp3+ regulatory T cell function. *Science* (2008) 322:271–5. doi: 10.1126/science.1160062
121. Walker LSK. EFIS Lecture: Understanding the CTLA-4 checkpoint in the maintenance of immune homeostasis. *Immunol Lett.* (2017) 184:43–50. doi: 10.1016/j.imlet.2017.02.007
122. Walker LSK, Sansom DM. The emerging role of CTLA4 as a cell-extrinsic regulator of T cell responses. *Nat Rev Immunol.* (2011) 11:852–63. doi: 10.1038/nri3108
123. Zhao DM, Thornton AM, DiPaolo RJ, Shevach EM. Activated CD4+CD25+ T cells selectively kill B lymphocytes. *Blood* (2006) 107:3925–32. doi: 10.1182/blood-2005-11-4502
124. Wu H, Chen Y, Liu H, Xu L, Teuscher P, Wang S, et al. Follicular regulatory T cells repress cytokine production by follicular helper T cells and optimize IgG responses. *Eur J Immunol.* (2016) 1152–61. doi: 10.1002/eji.201546094
125. Kawamoto S, Maruya M, Kato LM, Suda W, Atarashi K, Doi Y, et al. Foxp3+ T cells regulate immunoglobulin A selection and facilitate diversification of bacterial species responsible for immune homeostasis. *Immunity* (2014) 41:152–65. doi: 10.1016/j.immuni.2014.05.016
126. Sage PT, Tan CL, Freeman GJ, Haigis M, Sharpe AH. Defective TFH cell function and increased TFR cells contribute to defective antibody production in aging. *Cell Rep.* (2015) 12:163–71. doi: 10.1016/j.celrep.2015.06.015
127. Ding Y, Li J, Yang PA, Luo B, Wu Q, Zajac AJ, et al. Interleukin-21 promotes germinal center reaction by skewing the follicular regulatory T cell to follicular helper T cell balance in autoimmune BXD2 mice. *Arthritis Rheumatol.* (2014) 66:2601–12. doi: 10.1002/art.38735
128. Jang E, Cho WS, Cho M-L, Park H-J, Oh H-J, Kang SM, et al. Foxp3+ regulatory T cells control humoral autoimmunity by suppressing the development of long-lived plasma cells. *J Immunol.* (2011) 186:1546–53. doi: 10.4049/jimmunol.1002942
129. Kinnunen T, Chamberlain N, Morbach H, Choi J, Kim S, Craft J, et al. Accumulation of peripheral autoreactive B cells in the absence of functional human regulatory T cells. *Blood* (2013) 121:1595–603. doi: 10.1182/blood-2012-09-457465

130. Ludwig-Portugall I, Hamilton-Williams EE, Gottschalk C, Kurts C. Cutting edge: CD25+ regulatory T cells prevent expansion and induce apoptosis of B cells specific for tissue autoantigens. *J Immunol.* (2008) 181:4447–51. doi: 10.4049/jimmunol.181.7.4447
131. Seo S, Fields ML, Buckler JL, Reed AJ, Mandik-Nayak L, Nish SA, et al. The impact of T helper and T regulatory cells on the regulation of anti-double-stranded DNA B cells. *Immunity* (2002) 16:535–46. doi: 10.1016/S1074-7613(02)00298-4
132. Buzryn D, Benoist C, Mathis D. Regulatory T cells in nonlymphoid tissues. *Nat Immunol.* (2013) 14:1007–13. doi: 10.1038/ni.2683
133. Campbell DJ, Koch MA. Phenotypical and functional specialization of FOXP3+ regulatory T cells. *Nat Rev Immunol* (2011) 11:119–30. doi: 10.1038/nri2916
134. Liston A, Gray DHD. Homeostatic control of regulatory T cell diversity. *Nat Rev Immunol.* (2014) 14:154–65. doi: 10.1038/nri3605.
135. Vignali D, Collison L, Workman C. How regulatory T cells work. *Nat Rev Immunol.* (2008) 8:523–32. doi: 10.1038/nri2343
136. Sayin I, Radtke AJ, Vella LA, Jin W, Wherry EJ, Buggert M, et al. Spatial distribution and function of T follicular regulatory cells in human lymph nodes. *J Exp Med.* (2018) 215:1531–42. doi: 10.1084/jem.20171940
137. Lim HW, Hillsamer P, Kim CH. Regulatory T cells can migrate to follicles upon T cell activation and suppress GC-Th cells and GC-Th cell-driven B cell responses. *J Clin Invest.* (2004) 114:1640–9. doi: 10.1172/JCI200422325
138. Lim HW, Hillsamer P, Banham AH, Kim CH. Cutting edge: direct suppression of B cells by CD4+ CD25+ regulatory T cells. *J Immunol.* (2005) 175:4180–3. doi: 10.4049/jimmunol.175.7.4180
139. Reboldi A, Cyster JG. Peyer's patches: organizing B-cell responses at the intestinal frontier. *Immunol Rev.* (2016) 271:230–45. doi: 10.1111/imr.12400
140. Macpherson AJ, Geuking MB, Slack E, Hapfelmeier S, McCoy KD. The habitat, double life, citizenship, and forgetfulness of IgA. *Immunol Rev.* (2012) 245:132–46. doi: 10.1111/j.1600-065X.2011.01072.x
141. Craig SW, Cebra JJ. Peyer's patches: an enriched source of precursors for IgA-producing immunocytes in the rabbit. *J. Exp. Med.* (1971) 134:188–200.
142. Pabst O. New concepts in the generation and functions of IgA. *Nat Rev Immunol.* (2012) 12:821–32. doi: 10.1038/nri3322
143. Rey J, Garin N, Spertini F, Corthésy B. Targeting of secretory IgA to Peyer's patch dendritic and T cells after transport by intestinal M cells. *J Immunol.* (2004) 172:3026–33. doi: 10.4049/JIMMUNOL.172.5.3026
144. Fagarasan S, Muramatsu M, Suzuki K, Nagaoka H, Hiai H, Honjo T. Critical roles of activation-induced cytidine deaminase in the homeostasis of gut flora. *Science* (2002) 298:1424–7. doi: 10.1126/science.1077336
145. Suzuki K, Meek B, Doi Y, Muramatsu M, Chiba T, Honjo T, et al. Aberrant expansion of segmented filamentous bacteria in IgA-deficient gut. *Proc Natl Acad Sci USA.* (2004) 101:1981–6. doi: 10.1073/pnas.0307317101
146. Fadlallah J, El Kafsi H, Sterlin D, Juste C, Parizot C, Dorgham K, et al. Microbial ecology perturbation in human IgA deficiency. *Sci Transl Med.* (2018) 10:eaa1217. doi: 10.1126/scitranslmed.aan1217
147. Bergqvist P, Gärdby E, Stensson A, Bemark M, Lycke NY. Gut IgA class switch recombination in the absence of CD40 does not occur in the lamina propria and is independent of germinal centers. *J Immunol.* (2006) 177:7772–83. doi: 10.4049/JIMMUNOL.177.11.7772
148. Bunker JJ, Flynn TM, Koval JC, Shaw DG, Meisel M, McDonald BD, et al. Innate and adaptive humoral responses coat distinct commensal bacteria with immunoglobulin A. *Immunity* (2015) 43:541–53. doi: 10.1016/j.immuni.2015.08.007
149. Gärdby E, Wråmmert J, Schön K, Ekman L, Leanderson T, Lycke N. Strong differential regulation of serum and mucosal IgA responses as revealed in CD28-deficient mice using cholera toxin adjuvant. *J Immunol.* (2003) 170:55–63. doi: 10.4049/jimmunol.170.1.55
150. Wei M, Shinkura R, Doi Y, Maruya M, Fagarasan S, Honjo T. Mice carrying a knock-in mutation of Aicda resulting in a defect in somatic hypermutation have impaired gut homeostasis and compromised mucosal defense. *Nat Immunol.* (2011) 12:264–70. doi: 10.1038/ni.1991
151. Hirota K, Turner J-E, Villa M, Duarte JH, Demengeot J, Steinmetz OM, et al. Plasticity of Th17 cells in Peyer's patches is responsible for the induction of T cell-dependent IgA responses. *Nat Immunol.* (2013) 14:372–9. doi: 10.1038/ni.2552
152. Tsuji M, Komatsu N, Kawamoto S, Suzuki K, Kanagawa O, Honjo T, et al. Preferential generation of follicular B helper T cells from Foxp3+ T Cells in Gut Peyer's Patches. *Science* (2009) 323:1488–92. doi: 10.1126/science.1169152
153. Proietti M, Cornacchione V, Rezzonico Jost T, Romagnani A, Faliti CE, Perruzza L, et al. ATP-gated ionotropic P2X7 receptor controls follicular T helper cell numbers in Peyer's patches to promote host-microbiota mutualism. *Immunity* (2014) 41:789–801. doi: 10.1016/j.immuni.2014.10.010
154. Fei Teng A, Klinger CN, Felix KM, Tran NL, Umesaki Y, Joyce Wu Correspondence HJ, et al. Gut microbiota drive autoimmune arthritis by promoting differentiation and migration of Peyer's patch T follicular helper. *Cells* (2016) 44:875–88. doi: 10.1016/j.immuni.2016.03.013
155. Kato LM, Kawamoto S, Maruya M, Fagarasan S. Gut TFH and IgA: key players for regulation of bacterial communities and immune homeostasis. *Immunol Cell Biol.* (2014) 92:49–56. doi: 10.1038/icb.2013.54
156. Ramiscal RR, Vinuesa CG. T-cell subsets in the germinal center. *Immunol Rev.* (2013) 252:146–55. doi: 10.1111/imr.12031
157. Georgiev H, Ravens I, Papadogianni G, Halle S, Malissen B, Loots GG, et al. Shared and unique features distinguishing follicular T helper and regulatory cells of peripheral lymph node and Peyer's patches. *Front Immunol.* (2018) 9:714. doi: 10.3389/fimmu.2018.00714
158. Cong Y, Feng T, Fujihashi K, Schoeb TR, Elson CO. A dominant, coordinated T regulatory cell-IgA response to the intestinal microbiota. *Proc Natl Acad Sci USA.* (2009) 106:19256–61. doi: 10.1073/pnas.0812681106
159. Round JL, Mazmanian SK. The gut microbiota shapes intestinal immune responses during health and disease. *Nat Rev Immunol.* (2009) 9:313–23. doi: 10.1038/nri2515
160. Khosravi A, Yá Ñ, Ez A, Price JG, Chow A, Merad M, Goodridge HS, et al. Gut microbiota promote hematopoiesis to control bacterial infection. *Cell Host Microbe* (2014) 15:374–81. doi: 10.1016/j.chom.2014.02.006
161. Vieira Q, Teixeira MM, Souza DG, Soares AC, Pinho V, Nicoli JR, et al. Germfree mice control pulmonary bacterial infection in inflammatory response and ability to transient TLR activation restores transient TLR activation restores inflammatory response and ability to control pulmonary bacterial infection in germfree mice. *J Immunol Ref.* (2018) 188:1411–20. doi: 10.4049/jimmunol.1101682
162. Kim M, Qie Y, Park J, Kim CH. Gut microbial metabolites fuel host antibody responses. *Cell Host Microbe* (2016) 20:202–14. doi: 10.1016/j.chom.2016.07.001
163. Wu W, Sun M, Chen F, Cao AT, Liu H, Zhao Y, et al. Microbiota metabolite short-chain fatty acid acetate promotes intestinal IgA response to microbiota which is mediated by GPR43. *Mucosal Immunol.* (2017) 10:946–56. doi: 10.1038/mi.2016.114

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2018 Stebegg, Kumar, Silva-Cayetano, Fonseca, Linterman and Graca. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



Control of the Germinal Center by Follicular Regulatory T Cells During Infection

Brodie Miles¹ and Elizabeth Connick^{2*}

¹ Vaccine and Gene Therapy Institute, Oregon Health & Science University, Beaverton, OR, United States, ² Division of Infectious Diseases, University of Arizona, Tucson, AZ, United States

OPEN ACCESS

Edited by:

Maria Pia Cicalese,
San Raffaele Scientific Institute
(IRCCS), Italy

Reviewed by:

Nabila Seddiki,
Vaccine Research Institute (VRI),
France
Lisa S. Westerberg,
Karolinska Institutet (KI), Sweden

*Correspondence:

Elizabeth Connick
connicke@deptofmed.arizona.edu

Specialty section:

This article was submitted to
T Cell Biology,
a section of the journal
Frontiers in Immunology

Received: 06 June 2018

Accepted: 01 November 2018

Published: 20 November 2018

Citation:

Miles B and Connick E (2018) Control
of the Germinal Center by Follicular
Regulatory T Cells During Infection.
Front. Immunol. 9:2704.
doi: 10.3389/fimmu.2018.02704

Follicular regulatory T cells (Tfr) are a unique subset of CD4 T cells that control and impact adaptive immune responses in the lymphoid follicles and germinal centers (GC). Since their relatively recent discovery, several studies have revealed that Tfr interact with other cells within this niche and shape ensuing responses. Recent advances defining the functional and developmental characteristics of Tfr have revealed key characteristics of Tfr differentiation, GC recruitment and retention, and regulatory properties. Further, Tfr shape the GC response and balance tolerance through interactions with Tfh, by modifying Tfh number, diversity and function, as well as with B cells. Mechanisms by which Tfr regulate the GC include cell-to-cell interactions with Tfh and B cells, as well as altering their environment through cytokine production and sequestration. Tfr have been shown to have a diverse T cell receptor (TCR) repertoire and can be specific for immunizing agents, demonstrating a potential role in vaccine development. Due to these important characteristics and functions, Tfr play a major role in immune tolerance, response to infection, and vaccine efficacy.

Keywords: follicular regulatory T cells, germinal center, immune regulation, infection, vaccination

CHARACTERISTICS AND FUNCTIONS OF TFR

Follicular regulatory T cells (Tfr) are a subset of regulatory T cells (Treg) that suppress follicular T helper cells (Tfh) and B cells. Tfr were first identified in mice and shown to play a crucial role in the germinal center (GC) response and antibody production (1–3). Tfr express high levels of CXCR5, which directs them to follicles and GCs (3). Similar to Tfh, Tfr require ICOS and CD28 to promote development and maintenance (2), and are inhibited by high levels of PD-1 (4) and IL-2 (5). Tfr require T cell co-stimulation for growth and development, as CD28 deletion results in nearly total loss of Tfr in spleen and lymph node after immunization (2).

Tfr express high levels of the transcription factor Bcl-6 (1). Uniquely, Tfr are able to co-express the transcription factors Bcl-6 and Blimp-1, although these factors are typically believed to be part of a negative feedback loop (1, 3). Loss of Bcl-6 results in almost a complete loss of Tfr, however, almost paradoxically, Blimp1 is the master regulator of Tfr function (2). Further, NFAT-2 is a critical Tfr transcription factor that promotes Tfr differentiation and retention in the GC and lymph node follicles through upregulation of CXCR5 expression (6). Lastly, SLAM-associated protein (SAP) is increased in fully differentiated Tfr and plays a critical role in Tfr maintenance by mediating the interactions of Tfr with B cells in the GC, as SAP deletion results in the loss of GC B cells and defective GCs (2).

Similar to Tfh, Tfr require the GC microenvironment and GC B cells for their development and function, as they are dependent on ICOS-ICOSL interactions for regulatory function and expansion. However, although phenotypically similar to Tfh, Tfr originate from CD25⁺ Foxp3⁺ Treg precursors while Tfh originate from CD4⁺Foxp3⁻ T cell precursors (1, 2). Antigen presenting cells play a definitive role in development and maturation of Tfr. B cells in the GC of lymph node follicles promote optimal Tfr growth and differentiation. In addition, dendritic cell (DC) subsets in mucosal and peripheral tissue sites promote Tfr development. Mice that were depleted of DCs had a significant reduction in the proportion of Tfr (7). This suggests that Tfr development and maintenance can be highly tissue specific and require multiple cell subsets and environmental cues. In addition, Tfr express potentially autoreactive TCR repertoires leading to their ability to suppress autoimmunity, in contrast to Tfh, which arise from antigen-responsive TCRs allowing for promotion of antibody responses (8).

The GC microenvironment is important to driving follicular T cell differentiation while maintaining cell phenotype and function. After their differentiation and antigen imprinting, Tfr either enter circulation to become memory Tfr or localize to the B cell zone to interact with B cells and Tfh. Studies in mice have demonstrated that memory Tfh lose expression of CXCR5, PD-1, and Bcl-6 after adoptive transfer into naïve hosts (9). However, circulating Tfh from human donors have been cultured up to 3 weeks without stimulation and maintained CXCR5 and PD-1 expression (10). Importantly, Tfr were included within the Tfh subset in both of these analyses. Factors that promote Tfr egress from the follicle are unknown. Receptor mediated endocytosis of CXCR5 has been described for B cells (11). It is possible that CXCR5 is downregulated transiently on T cells in the context of high levels of CXCL13 in the follicle, thereby enabling these cells to exit the follicle before CXCR5 is re-expressed. In addition, Tfr may upregulate other receptors that promote migration to other sites. For example, in Sage et al.'s study (7), peripheral blood Tfr were enriched for CXCR3, which may direct Tfr to other sites outside of the lymph nodes. When Tfr migrate out of the follicle, they have similar CXCR5 levels, but diminished ICOS expression and PD-1 expression, indicating the importance of the GC microenvironment for development and maintenance of Tfr phenotype (7). Adding more complexity, a recent study characterized a subset of Tfr lacking CD25 expression, thus being independent of IL-2 signaling and allowing for increased CXCR5 and Bcl-6 expression (12).

TFR REGULATION OF TFH

Similar to Treg, Tfr are able to suppress immune responses through a variety of mechanisms. The suppressive function of Tfr was initially shown in mouse adoptive transfer experiments where fully differentiated Tfr were shown to potently suppress antigen-specific antibody production and GC responses (2). Further, isolated Tfr cultured with GC B cells from immunized mice showed that Tfr substantially diminish IgG output *in vitro*

(4). Similar to Treg, Tfr utilize mechanisms to suppress Tfh and B cells (described below) such as downregulation of co-stimulatory molecules, cytokine production, and direct physical disruption, while metabolic disruption and cytolytic functions remain mostly unexplored.

One of the key regulatory effector molecules of Tfr is CTLA-4. CTLA-4 has been shown to control Foxp3⁺ Treg functions and act as a co-inhibitory molecule to dampen immune responses by preventing CD28-B7 co-stimulatory interactions (13, 14). Through genetic deletions in mouse models, CTLA-4 expression by Tfr has been shown to play a crucial role in Tfh differentiation and functional responses. CTLA-4 expression on Tfr potently suppresses Tfh generation, differentiation, and subsequent B cell responses (15, 16). CTLA-4 expression in Treg controls Tfh antigen-specific expansion and Tfh cell numbers (16). It should be noted, however, that global deletion of CTLA-4 altered Tfh numbers rather than simply Tfr CTLA-4 levels (15). Blockade of CTLA-4 resulted in spontaneous Tfh differentiation and large GC expansion in a CD28-dependent manner, as CD28 heterozygosity also reduced Tfh differentiation while leaving other facets of T cell activation unaltered (17).

Mouse models have also demonstrated that expression of PD-1 plays a large role in the function of Tfr, as similar to most cell types engagement of PD-1 leads to loss of effector function and exhaustion. PD-1 expression on Tfr markedly reduced their ability to suppress Tfh function, while PD-1 deficiency led to heightened suppressive ability (4). In a study using adoptive transfer of OT-II cells into Cd3e-deficient mice (i.e., mice with abnormally low levels of lymphocytes in the blood), which causes increases of Tfh and impaired GC responses, the addition of Treg restored normal Tfh cell numbers, B cell distribution within the GC, and somatic hypermutation rates (18). Further, PD-L1 deficient mice have higher percentages of Tfr and increased Blimp-1 and Bcl-6 expression, demonstrating that PD-1 signals could inhibit Tfr differentiation and accumulation (4). Tfr responses can also be manipulated by Tfh function, as IL-21 serves as negative feedback for downregulation of CD25 (and IL-2 responsiveness) through Bcl-6 expression (19). Human Tfr reduce IL-21 and IL-4 production by Tfh in an *ex vivo* HIV infection model by a mechanism that is contact dependent (20). Thus, Tfr may regulate Tfh production of IL-21 both to limit the GC response and also to prevent loss of their own effector functions.

The dynamics of Tfr and Tfh interactions can vary based on the microenvironment and circumstances of immune responses. Tfr were shown to accumulate in number and proportion to Tfh in untreated, chronically HIV-infected individuals' lymph nodes (20). Similarly, increases in percentages of circulating cells with a follicular regulatory phenotype are seen in individuals with untreated chronic hepatitis B infection (21, 22). In an *ex vivo* HIV infection model, Tfr led to a decrease in Tfh ICOS expression and inhibited rates of Tfh proliferation (20). Depletion of Tfr in mice did not alter Tfh and GC B cell populations upon immunization, however, the quality of the GC response was diminished as antigen-specific antibody responses were altered and IgG production was reduced (23). Interestingly, Foxp3 depletion in mice was shown to compromise influenza-specific

Tfh responses due to suppression of Tfh differentiation via increased IL-2 availability (24), thereby demonstrating a positive role for Tfr in favorable Tfh responses. Rather than absolute numbers of Tfr and Tfh, the ratio of Tfr to Tfh in the GC is thought to be critical to generating immune responses (4), as well as regulating autoimmunity (25).

Tfr have been demonstrated to allow initial B cell activation, but to physically disrupt Tfh-B cell interactions and thereby limit GC effector cell function (26). RNAseq transcriptome analysis revealed that global gene expression does not differ substantially between Tfh that have been suppressed by Tfr and (unsuppressed) active Tfh populations. Further transcriptome analysis revealed that Tfr suppressed Tfh expression of key effector molecules such as IL-4, IL-21, IL-10, and CD28, but did not alter expression of key transcription factors such as Bcl-6 or CXCR5 expression. This, as the authors suggest, demonstrates that Tfr suppress Tfh in a manner that allows them to retain their Tfh differentiation and maintenance profile, but inhibit upregulation of their specific effector molecules.

TFR REGULATION OF B CELLS

Tfr were first defined as a specialized subset of Treg that suppress B cell responses (2). Whereas the suppressive effects of Tfr on Tfh function are well established, few studies have investigated whether Tfr have a direct suppressive effect on B cells. Using human tonsil cells, Lim et al. demonstrated that CD4+CD25+ cells directly inhibited B cell function and antibody responses and class switch recombination (27). Importantly, because suppression of GC B cell responses is unique to Tfr, and not mediated by CXCR5- Treg (2), presumably the activity observed by Lim et al. was mediated by Tfr. Deletion of CTLA-4 specifically in Treg in mice led to large increases in antibody production, suggesting an essential role for CTLA-4 in controlling antibody production (14). Further, another study has shown mice lacking Tfh/Tfr that receive CTLA-4 –deficient Tfh/Tfr through adoptive transfer developed Tfh expansions, increased antigen-specific antibody production, but no alterations in GC B cell numbers or co-stimulatory molecule expression (15). However, Treg-specific CTLA-4 deletion was shown to lead to decreased CD86 expression on follicular, albeit not GC, B cells (15). While this shows that Tfr function and Tfh/Tfr ratio play an essential role in GC B cell responses, it does not demonstrate a direct suppression of GC B cells by Tfr.

Regardless of whether Tfr inhibit B cells directly, or through loss of Tfh effector function, B cells which have been suppressed by Tfr display unique transcriptional profiles and altered metabolic states that are associated with inability to activate Tfh and to perform class switch recombination. IgG2b transcripts were elevated, while IgG1, IgG2a, and IgA transcripts were lowered in suppressed B cell populations, as well as a few key effector molecules, such as the cytidine deaminase AID, however, most gene signatures were intact suggesting that Tfr suppress the expression of specific effector molecules (26). More profound gene expression changes in suppressed B cells were seen in B cell metabolic pathways, such as glycolysis, and upstream mediators including mTOR. Further, suppressed B cells also displayed defects in various metabolic and anabolic processes,

including suppression of glycolysis. Glycolysis is crucial for antibody production (28), and interestingly this regulation of glycolysis was found to be independent of B cell proliferation (26). Intriguingly, B cell suppression persisted even in the absence of Tfr, but was reversed by IL-21.

B cells produce the pro-inflammatory chemokines CCL3 and CCL4 during GC formation and response. Tfr respond to this chemokine production by infiltrating the GC, interacting with B cells, and suppressing numbers of self-reactive B cell clones within the GC (29). PD-L1 expression on GC B cells has been shown to inhibit Tfh function based on their high expression of PD-1 and as a result preventing Tfh from optimally stimulating antibody production (30), although this study did not address if PD-L1 similarly alters Tfr function. In mesenteric lymph nodes from individuals without known infection, Tfr were found to occupy the border of the T cell zone and B cell follicle and express relatively lower levels of PD-1 than Tfh (31). Despite appearing in the GC at low frequency compared to Tfh and expressing low levels of PD-1, Tfr were able to potently suppress antibody production *in vitro* (31). Further demonstrating Tfr localization to the GC is a critical element to GC function, in patients with the autoimmune disease Sjogrens syndrome, Tfr are excluded from ectopic GCs, thus physically separating them from GC B cells and potentially contributing to autoimmunity (32).

After immediate activation of the GC response and B cell expansion, Tfr proportionally decrease, but as responses wane and the GC reaction is resolved, Tfr return to baseline levels. Within the B cell follicle, Tfr are highly motile when receiving antigenic stimulation from B cells (33). This study also showed that blocking CTLA-4-B7 interactions leads to reduced T cell interaction, reduced dendritic cell interaction, and increased T cell proliferation including Tfr during immune priming. In mice, it is well documented that Tfh and Tfr are completely dependent on the GC for their maintenance (34). However, a study of human patients receiving organ transplants showed that treatment with rituximab, which results in loss of GC B cells (anti-CD20 antibody), did not prevent the loss of Tfr populations (35). Thus Tfr may not require an ongoing GC response for maintenance in humans. Alternatively, Tfr interactions with certain cell populations in the blood or tissues such as memory or naïve B cells, as well as follicular dendritic cells, may be able to fully support Tfr maintenance in the absence of the GC. This suggests that Tfr may also be maintained in the blood of humans in a similar fashion seen with functional Tfh populations (10).

An interesting aspect of Tfr and B cell interactions apart from immune regulation are recent studies that demonstrate Tfr promote GC B cell responses. In a mouse model using lymphocytic choriomeningitis virus (LCMV), IL-10-producing Tfr promote GC formation, dark zone morphology, and B cell FOXO1 expression (36). Tfh production of IL-9 has been shown to promote cell cycling and generation of memory-precursor B cells, however, this study did not address whether this is also influenced by either IL-9 production by Tfr or IL-9 sequestering by Tfr (37). As Tfr are believed to prevent excessive antigen-driven expansion and promote clonal selection, it would be important to understand if Tfr selectively inhibit GC B cells while helping to promote memory or plasma cell function.

ROLE OF TFR IN RESPONSE TO INFECTION

As the master regulators of GC B cell function, Tfh function, and antibody production Tfr play a crucial role in responses to microbial infections. In mouse models of acute LCMV infection, IL-10-producing Tfr were elicited and shown to support GC responses (36). In an influenza infection model in mice, high IL-2 levels at the time of infection prevented Tfr development in a Blimp1-dependent manner (5). After resolution of infection, Treg downregulated CD25 expression, differentiated into Tfr cells, and migrated into the follicles to prevent expansion of self-reactive B cell clones (5). This is in accordance with the aforementioned study that displayed loss of CD25 on Treg promotes expression of CXCR5 and Bcl-6 leading to the development of a Tfr subset (18).

Optimal GC reactions and effector B cell responses are crucial to the resolution of microbial infections. Thus, T cell help and programming of these responses play a large role in the outcome of these responses. Optimal Tfh function is required to promote adequate B cell function that leads to diverse, high avidity, antigen-specific antibody responses. In chronically HIV-infected individuals, Tfh populations expand and provide inadequate help to B cells (7, 38). These deficiencies are manifested in defects in Tfh proliferation, ICOS expression, IL-21 production, and signaling to stimulate Ig production (30). The extent to which Tfr control these Tfh responses is a current area of great interest. IL-6 promotes Tfh function and alleviates chronic viral infection in mice, due in part to inhibiting TGF- β -dependent generation of regulatory T cells and increased Bcl-6 expression in Tfh (39). As boosted Tfh function and potential loss of immune regulation helped to resolve chronic infection in mice, Tfr expanded and reduced the quality of Tfh effector function in chronically HIV-infected humans and SIV-infected rhesus macaques (20). Further, viral infection appeared to drive Tfr differentiation in culture (20) and Tfr were shown to be highly permissive to HIV infection (40). Thus, pathogens may have the ability to program Tfr activity and numbers and alter the GC response. These interactions could lead to inadequate GC responses independent of Tfh and B cell interactions with Tfr and result in suboptimal responses and resolution of infection.

Tfr also have complex interactions with microbiota and commensals in the gastrointestinal tract. Foxp3+ cells in the GCs of Peyer's patches have been shown to regulate gut microbiota diversity and IgA selection, which in turn leads to expansion of Foxp3+ cells in a symbiotic regulatory loop (41). During inflammatory responses in the intestinal tract, Foxp3+ cells are stabilized and have enhanced suppressive capacity (42). These Foxp3+ cells also express ROR γ t, as well as signature regulatory receptors such as CLTA-4 and GITR, and are central to immune tolerance and limiting inflammation in the intestinal mucosa (42).

The protein kinase mTOR, which senses and integrates environmental cues to impact cellular function during viral infections, was recently characterized in Tfr after acute LCMV infection in mice (43). mTORC1 was found to be essential for Tfr differentiation during viral infection by activating STAT-3 to

promote the TCF-1/Bcl-6 transcriptional axis to launch the Tfr transcriptional program. Further, it was recently demonstrated in a Bcl-6/Foxp3-deficient mouse model that loss of Tfr led to excessive lymphocyte infiltration and antibody deposition resulting in autoimmunity (44). Even slight imbalances and decreased ratios of Tfr to Tfh correlate with autoimmunity and could potentially be used as a clinical diagnostic for excessive immune activation and inadequate GC responses (25).

MEMORY TFR AND VACCINE DEVELOPMENT

The extent to which Tfr have memory properties and display recall function for self and foreign antigens is still unclear. PD-L1 expression on DCs limited Tfr differentiation and subsequent immune responses, suggesting that PD-1 interactions can limit Tfr priming and memory differentiation (45). Due to the complexity of sharing properties with Treg and Tfh, it is unclear if Tfr can become antigen specific and the implications this plays in vaccine development. Tfh are clearly understood to be specific for immunizing and infectious agents (34, 38, 46), whereas Treg have TCRs skewed toward recognition of self-antigens providing their role in autoimmunity (47, 48). One study has shown that Tfr are able to develop from naïve (Foxp3-) T cells in a PD-L1-dependent manner and that these cells can be specific for various immunizing agents used in a mouse model of vaccinations (49). Thus, antigen and Tfr TCR engagement likely plays a role in Tfr memory development and maintenance and therefore could be useful in modulating responses to vaccines.

Circulating Tfr are memory-like, displaying recall responses and similar effector functions to GC Tfr, but interestingly only require DCs and not B cells for differentiation (7). In addition, circulating memory Tfr expand and increase activity in GVHD patients given daily IL-2 therapy, which promotes immune tolerance (50). As circulating Tfr and lymph node-resident Tfr have differing levels of CXCR5 and ICOS expression, these subsets may have different gene expression patterns and may have different requirements for harnessing their full vaccine potential. A recent study showed that GC B cells that proliferate and produce high affinity antibodies receive help from T cells in the light zone that promotes their DNA replication and limits the duration of the S phase by regulating replication fork progression (51). It is currently unclear whether Tfr provide help to GC B cells to promote the speed of B cell replication and activation as well as high affinity selection, or limit Tfh activity to balance T cell help to B cells. The loss of STAT-3 expression in immunized mice led to a reduction of Tfr and increased levels of specific IgG1 and IgG2b antibodies, however, Tfh levels were unaffected by loss of either STAT-3 or Tfr (52).

The generation of broadly neutralizing antibodies (bnab) is a major goal of HIV vaccine development, among other infectious diseases. Most HIV vaccine development programs aim to harness the natural conditions that favor bnab production. A higher frequency of circulating memory Tfh and PD-1-expressing Treg (presumably circulating Tfr) was detected in HIV-infected individuals who make bnabs (53). In SHIV-infected rhesus

macaques, lowered Foxp3 expression correlated with increased bnab production (54). This suggests that Tfr play a negative role in antibody diversity and strongly merits investigation in development of vaccines aimed at generating bnabs. IL-21 treatments are able to override Tfr function and promote Tfh and B cell functions (26). The role of Tfr in regulating antibody affinity or diversity has yet to be fully elucidated. While Tfr have a role in suppressing high and low affinity antibody production (1), it remains to be seen if Tfr control antibody diversity through affinity maturation.

CONCLUSIONS

Tfr are a unique subset of T helper cells that play a critical role in controlling immune responses due to their functions in the GC response. They fine tune the immune response and constantly

interact with Tfh and B cell populations, thereby influencing the quantity and quality of their functions. There is still much work to do to define their mechanisms of action and role in the germinal center response. A better understanding of Tfr could lead to novel therapeutic and vaccine strategies to treat many infectious diseases.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

This work was funded by NIH/NIAID grant R01 AI096966 to EC.

REFERENCES

- Chung Y, Tanaka S, Chu F, Nurieva RI, Martinez GJ, Rawal S, et al. Follicular regulatory T cells expressing Foxp3 and Bcl-6 suppress germinal center reactions. *Nat Med.* (2011) 17:983. doi: 10.1038/nm.2426
- Linterman MA, Pierson W, Lee SK, Kallies A, Kawamoto S, Rayner TF, et al. Foxp3+ follicular regulatory T cells control the germinal center response. *Nat Med.* (2011) 17:975–82. doi: 10.1038/nm.2425
- Wollenberg I, Agua-Doce A, Hernández A, Almeida C, Oliveira VG, Faro J, et al. Regulation of the germinal center reaction by Foxp3+ follicular regulatory T cells. *J Immunol.* (2011) 187:4553–60. doi: 10.4049/jimmunol.1101328
- Sage PT, Francisco LM, Carman CV, Sharpe AH. The receptor PD-1 controls follicular regulatory T cells in the lymph nodes and blood. *Nat Immunol.* (2013) 14:152. doi: 10.1038/ni.2496
- Botta D, Fuller MJ, Marquez-Lago TT, Bachus H, Bradley JE, Weinmann AS, et al. Dynamic regulation of T follicular regulatory cell responses by interleukin 2 during influenza infection. *Nat Immunol.* (2017) 18:1249. doi: 10.1038/ni.3837
- Vaeth M, Müller G, Stauss D, Dietz L, Klein-Hessling S, Serfling E, et al. Follicular regulatory T cells control humoral autoimmunity via NFAT2-regulated CXCR5 expression. *J Exp Med.* (2014) 211:545–61. doi: 10.1084/jem.20130604
- Sage PT, Alvarez D, Godec J, Von Andrian UH, Sharpe AH. Circulating T follicular regulatory and helper cells have memory-like properties. *J Clin Invest.* (2014) 124:5191–204. doi: 10.1172/JCI76861
- Maceiras AR, Almeida SCP, Mariotti-Ferrandiz E, Chaara W, Jebbawi F, Six A, et al. T follicular helper and T follicular regulatory cells have different TCR specificity. *Nat Commun.* (2017) 8:15067. doi: 10.1038/ncomms15067
- Weber JP, Fuhrmann F, Hutloff A. T-follicular helper cells survive as long-term memory cells. *Eur J Immunol.* (2012) 42:1981–8. doi: 10.1002/eji.201242540
- Locci M, Havenar-Daughton C, Landais E, Wu J, Kroenke MA, Arlehamm CL, et al. Human circulating PD-1+ CXCR3– CXCR5+ memory Tfh cells are highly functional and correlate with broadly neutralizing HIV antibody responses. *Immunity* (2013) 39:758–69. doi: 10.1016/j.immuni.2013.08.031
- Cagigi A, Mowafi F, Dang LVP, Tenner-Racz K, Atlas A, Grutzmeier S, et al. Altered expression of the receptor-ligand pair CXCR5/CXCL13 in B cells during chronic HIV-1 infection. *Blood* (2008) 112:4401–10. doi: 10.1182/blood-2008-02-140426
- Wing JB, Kitagawa Y, Locci M, Hume H, Tay C, Morita T, et al. A distinct subpopulation of CD25– T-follicular regulatory cells localizes in the germinal centers. *Proc Natl Acad Sci.* (2017) 05551. doi: 10.1073/pnas.1705551114
- Krummel MF, Allison JP. CD28 and CTLA-4 have opposing effects on the response of T cells to stimulation. *J Exp Med* (1995) 182:459–65. doi: 10.1084/jem.182.2.459
- Wing K, Onishi Y, Prieto-Martin P, Yamaguchi T, Miyara M, Fehervari Z, et al. CTLA-4 control over Foxp3+ regulatory T cell function. *Science* (2008) 322:271–5. doi: 10.1126/science.1160062
- Sage PT, Paterson AM, Lovitch SB, Sharpe AH. The coinhibitory receptor CTLA-4 controls B cell responses by modulating T follicular helper, T follicular regulatory, and T regulatory cells. *Immunity* (2014) 41:1026–39. doi: 10.1016/j.immuni.2014.12.005
- Wing JB, Ise W, Kurosaki T, Sakaguchi S. Regulatory T cells control antigen-specific expansion of Tfh cell number and humoral immune responses via the coreceptor CTLA-4. *Immunity* (2014) 41:1013–25. doi: 10.1016/j.immuni.2014.12.006
- Wang CJ, Heuts F, Ovcinnikovs V, Wardzinski L, Bowers C, Schmidt EM, et al. CTLA-4 controls follicular helper T-cell differentiation by regulating the strength of CD28 engagement. *Proc Natl Acad Sci.* (2015) 112:524–9. doi: 10.1073/pnas.1414576112
- Preite S, Baumjohann D, Foglierini M, Basso C, Ronchi F, Rodriguez BMF, et al. Somatic mutations and affinity maturation are impaired by excessive numbers of T follicular helper cells and restored by Treg cells or memory T cells. *Eur J Immunol.* (2015) 45:3010–21. doi: 10.1002/eji.201545920
- Jandl C, Liu SM, Cañete PE, Warren J, Hughes WE, Vogelzang A, et al. IL-21 restricts T follicular regulatory T cell proliferation through Bcl-6 mediated inhibition of responsiveness to IL-2. *Nat Commun.* (2017) 8:14647. doi: 10.1038/ncomms14647
- Miles B, Miller SM, Folkvord JM, Kimball A, Chamanian M, Meditz AL, et al. Follicular regulatory T cells impair follicular T helper cells in HIV and SIV infection. *Nat Commun.* (2015) 6:8608. doi: 10.1038/ncomms9608
- Chen L, Zhu C, Li F, Wang Y, Bao R, Cao Z, et al. Correlation between hepatic human males absent on the first (hMOF) and viral persistence in chronic hepatitis B patients. *Cell Biosci.* (2018) 8:14. doi: 10.1186/s13578-018-0215-5
- Wu X, Su Z, Cai B, Yan L, Li Y, Feng W, et al. Increased Circulating Follicular Regulatory T-Like Cells May Play a Critical Role in Chronic Hepatitis B Virus Infection and Disease Progression. *Viral Immunol.* (2018) 31:379–88. doi: 10.1089/vim.2017.0171
- Wu H, Chen Y, Liu H, Xu LL, Teuscher P, Wang S, et al. Follicular regulatory T cells repress cytokine production by follicular helper T cells and optimize IgG responses in mice. *Eur J Immunol.* (2016) 46:1152–61. doi: 10.1002/eji.201546094
- León B, Bradley JE, Lund FE, Randall TD, Ballesteros-Tato A. FoxP3+ regulatory T cells promote influenza-specific Tfh responses by controlling IL-2 availability. *Nat Commun.* (2014) 5:3495. doi: 10.1038/ncomms4495
- Fonseca VR, Romão VC, Agua-Doce A, Santos M, López-Presa D, Ferreira AC, et al. The ratio of blood T follicular regulatory cells to T follicular

- helper cells marks ectopic lymphoid structure formation while activated follicular helper T cells indicate disease activity in primary sjögren's syndrome. *Arthritis Rheumatol.* (2018) 70:774–84. doi: 10.1002/art.40424
26. Sage PT, Ron-Harel N, Juneja VR, Sen DR, Maleri S, Sungnak W, et al. Suppression by T FR cells leads to durable and selective inhibition of B cell effector function. *Nat Immunol.* (2016) 17:1436. doi: 10.1038/ni.3578
 27. Lim HW, Hillsamer P, Banham AH, Kim CH. Cutting edge: direct suppression of B cells by CD4+ CD25+ regulatory T cells. *J Immunol.* (2005) 175:4180–3. doi: 10.4049/jimmunol.175.7.4180
 28. Caro-Maldonado A, Wang R, Nichols AG, Kuraoka M, Milasta S, Sun LD, et al. Metabolic reprogramming is required for antibody production that is suppressed in anergic but exaggerated in chronically BAFF-exposed B cells. *J Immunol.* (2014) 192:3626–36. doi: 10.4049/jimmunol.1302062
 29. Benet Z, Wu R, Marthi M, Turner J, and Grigorova I. (2016). "B cell-intrinsic production of CCL3/4 promotes direct sampling of Germinal Center B cells by T follicular regulatory cells and limits non-foreign antigen specific B cell response". *Am Assoc Immunol.*
 30. Cubas RA, Mudd JC, Savoye A-L, Perreau M, Van Grevenynghe J, Metcalf T, et al. Inadequate T follicular cell help impairs B cell immunity during HIV infection. *Nat Med.* (2013) 19:494–9. doi: 10.1038/nm.3109
 31. Sayin I, Radtke AJ, Vella LA, Buggert M, Jin W, Wherry EJ, et al. "Human T follicular regulatory cells (Tfr) able to inhibit antibody production are enriched at the lymph node TB border and rare in germinal centers". *J Immunol.* (2017) 198:152.
 32. Pontarini E, Murray-Brown W, Croia C, Astorri E, Lucchesi D, Lepse N, et al. "OP0300 Enrichment of T follicular-helper cells (TFH) and exclusion of t follicular-regulatory cells (TFR) from ectoPIC germinal centers in salivary glands of sjogren9s syndrome patients". *Ann Rheumatic Dis.* (2017) 76:180. doi: 10.1136/annrheumdis-2017-eular.6121
 33. Matheu MP, Othy S, Greenberg ML, Dong TX, Schuijs M, Deswarte K, et al. Imaging regulatory T cell dynamics and CTLA4-mediated suppression of T cell priming. *Nat Commun.* (2015) 6:6219. doi: 10.1038/ncomms7219
 34. Baumjohann D, Preite S, Rebaldi A, Ronchi F, Ansel KM, Lanzavecchia A, et al. Persistent antigen and germinal center B cells sustain T follicular helper cell responses and phenotype. *Immunity* (2013) 38:596–605. doi: 10.1016/j.immuni.2012.11.020
 35. Wallin EF, Jolly EC, Suchánek O, Bradley JA, Espéli M, Jayne DR, et al. Human T-follicular helper and T-follicular regulatory cell maintenance is independent of germinal centers. *Blood* (2014) 124:2666–74. doi: 10.1182/blood-2014-07-585976
 36. Laidlaw BJ, Lu Y, Amezcua RA, Weinstein JS, Vander Heiden JA, Gupta NT, et al. Interleukin-10 from CD4+ follicular regulatory T cells promotes the germinal center response. *Sci Immunol.* (2017) 2:eaan4767. doi: 10.1126/sciimmunol.aan4767
 37. Wang Y, Shi J, Yan J, Xiao Z, Hou X, Lu P, et al. Germinal-center development of memory B cells driven by IL-9 from follicular helper T cells. *Nat Immunol.* (2017) 18:921. doi: 10.1038/ni.3788
 38. Lindqvist M, Van Lunzen J, Soghoian DZ, Kuhl BD, Ranasinghe S, Kranias G, et al. Expansion of HIV-specific T follicular helper cells in chronic HIV infection. *J Clin Invest.* (2012) 122:3271. doi: 10.1172/JCI64314
 39. Harker JA, Lewis GM, Mack L, Zuniga EI. Late interleukin-6 escalates T follicular helper cell responses and controls a chronic viral infection. *Science* (2011) 334:825–9. doi: 10.1126/science.1208421
 40. Miller SM, Miles B, Guo K, Folkvord J, Meditz AL, McCarter MD, et al. Follicular regulatory T cells are highly permissive to R5-tropic HIV-1. *J Virol.* (2017) 91:e00430–e00417. doi: 10.1128/JVI.00430-17
 41. Kawamoto S, Maruya M, Kato LM, Suda W, Atarashi K, Doi Y, et al. Foxp3+ T cells regulate immunoglobulin a selection and facilitate diversification of bacterial species responsible for immune homeostasis. *Immunity* (2014) 41:152–65. doi: 10.1016/j.immuni.2014.05.016
 42. Yang B, Hagemann S, Mamareli P, Lauer U, Hoffmann U, Beckstette M, et al. Foxp3+ T cells expressing RORγt represent a stable regulatory T-cell effector lineage with enhanced suppressive capacity during intestinal inflammation. *Mucosal Immunol.* (2016) 9:444. doi: 10.1038/mi.2015.74
 43. Xu L, Huang Q, Wang H, Hao Y, Bai Q, Hu J, et al. The Kinase mTORC1 Promotes the Generation and Suppressive Function of Follicular Regulatory T Cells. *Immunity* (2017) 47:538–551. e535. doi: 10.1016/j.immuni.2017.08.011
 44. Fu W, Liu X, Lin X, Feng H, Sun L, Li S, et al. Deficiency in T follicular regulatory cells promotes autoimmunity. *J Exp Med.* (2017) 215:20170901. doi: 10.1084/jem.20170901
 45. Sage PT, Schildberg FA, Sobel RA, Kuchroo VK, Freeman GJ, Sharpe AH. Dendritic Cell PD-L1 Limits Autoimmunity and Follicular T Cell Differentiation and Function. *J Immunol.* (2018) 200:2592–602. doi: 10.4049/jimmunol.1701231
 46. Fazilleau N, Mcheyzer-Williams LJ, Rosen H, Mcheyzer-Williams MG. The function of follicular helper T cells is regulated by the strength of T cell antigen receptor binding. *Nat Immunol.* (2009) 10:375. doi: 10.1038/ni.1704
 47. Jordan MS, Boesteanu A, Reed AJ, Petrone AL, Hohenbeck AE, Lerman MA, et al. Thymic selection of CD4+ CD25+ regulatory T cells induced by an agonist self-peptide. *Nat Immunol.* (2001) 2:301. doi: 10.1038/86302
 48. Hori S, Haury M, Coutinho A, Demengeot J. Specificity requirements for selection and effector functions of CD25+ 4+ regulatory T cells in anti-myelin basic protein T cell receptor transgenic mice. *Proc Natl Acad Sci.* (2002) 99:8213–8. doi: 10.1073/pnas.122224799
 49. Aloulou M, Carr EJ, Gador M, Bignon A, Liblau RS, Fazilleau N, et al. Follicular regulatory T cells can be specific for the immunizing antigen and derive from naive T cells. *Nature Commun.* (2016) 7:10579. doi: 10.1038/ncomms10579
 50. Kamihara Y, Forcade E, Koreth J, Kim HT, Liu H, Douchet I, et al. Low-Dose Interleukin-2 Therapy Activates Circulating T Follicular Regulatory Cells (cTFR) and Suppresses Circulating T Follicular Helper Cells (cTFH) in Patients with Chronic Gvhd. *Biol Blood Marrow Transplant.* (2018) 24:S72. doi: 10.1016/j.bbmt.2017.12.617
 51. Gitlin AD, Mayer CT, Oliveira TY, Shulman Z, Jones MJ, Koren A, et al. T cell help controls the speed of the cell cycle in germinal center B cells. *Science* (2015) 349:643–6. doi: 10.1126/science.aac4919
 52. Wu H, Xie MM, Liu H, Dent AL. Stat3 is important for follicular regulatory T cell differentiation. *PLoS ONE* (2016) 11:e0155040. doi: 10.1371/journal.pone.0155040
 53. Moody MA, Pedroza-Pacheco I, Vandergrift NA, Chui C, Lloyd KE, Parks R., et al. Immune perturbations in HIV–1-infected individuals who make broadly reactive neutralizing antibodies. *Science Immunol.* (2016) 1, aag0851. doi: 10.1126/sciimmunol.aag0851
 54. Yamamoto T, Lynch RM, Gautam R, Matus-Nicodemus R, Schmidt SD, Boswell KL, et al. (2015). Quality and quantity of TFH cells are critical for broad antibody development in SHIVAD8 infection. *Sci Trans Med.* (2015) 7:298ra120–298ra120. doi: 10.1126/scitranslmed.aab3964

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2018 Miles and Connick. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



CCR9 Expressing T Helper and T Follicular Helper Cells Exhibit Site-Specific Identities During Inflammatory Disease

Ilaria Cosorich^{1†}, Helen M. McGuire^{1†}, Joanna Warren¹, Mark Danta² and Cecile King^{1,2*}

¹ Department of Immunology, The Garvan Institute of Medical Research, Darlinghurst, NSW, Australia, ² St Vincent's Clinical School, University of NSW, Sydney, NSW, Australia

OPEN ACCESS

Edited by:

Shahram Salek-Ardakani,
Pfizer, United States

Reviewed by:

Valter R. Fonseca,
Universidade de Lisboa, Portugal
Georges Abboud,
University of Florida, United States

*Correspondence:

Cecile King
c.king@garvan.org.au

[†]These authors have contributed
equally to this work

Specialty section:

This article was submitted to
T Cell Biology,
a section of the journal
Frontiers in Immunology

Received: 29 August 2018

Accepted: 26 November 2018

Published: 04 January 2019

Citation:

Cosorich I, McGuire HM, Warren J,
Danta M and King C (2019) CCR9
Expressing T Helper and T Follicular
Helper Cells Exhibit Site-Specific
Identities During Inflammatory
Disease. *Front. Immunol.* 9:2899.
doi: 10.3389/fimmu.2018.02899

CD4⁺ T helper (Th) cells that express the gut homing chemokine receptor CCR9 are increased in the peripheral blood of patients with inflammatory bowel disease and Sjögren's syndrome and in the inflamed lesions of autoimmune diseases that affect the accessory organs of the digestive system. However, despite the important role of the GIT in both immunity and autoimmunity, the nature of CCR9-expressing cells in GIT lymphoid organs and their role in chronic inflammatory diseases remains unknown. In this study, we analyzed the characteristics of CCR9⁺ Th and T follicular helper (Tfh) cells in GIT associated lymphoid tissues in health, chronic inflammation and autoimmunity. Our findings reveal an association between the transcriptome and phenotype of CCR9⁺ Th in the pancreas and CCR9⁺ Tfh cells from GIT-associated lymphoid tissues. GIT CCR9⁺ Tfh cells exhibited characteristics, including a Th17-like transcriptome and production of effector cytokines, which indicated a microenvironment-specific signature. Both CCR9⁺ Tfh cells and CCR9⁺ Th cells from GIT-associated lymphoid tissues migrated to the pancreas. The expression of CCR9 was important for migration of both subsets to the pancreas, but Tfh cells that accumulated in the pancreas had downmodulated expression of CXCR5. Taken together, the findings provide evidence that CCR9⁺ Tfh cells and Th cells from the GIT exhibit plasticity and can accumulate in distal accessory organs of the digestive system where they may participate in autoimmunity.

Keywords: CCR9 C-C chemokine receptor type 9, T follicular helper, T helper, inflammation, GIT = gastrointestinal tract, autoimmunity

INTRODUCTION

T Cell Mediated Damage to Self-Tissue

It was demonstrated over four decades ago that experimental, partial depletion of T cells precipitates self-tissue destructive inflammation (1, 2). Subsequent to these studies, a T cell transfer model of colitis was described in rats and then in mice. These studies showed that transfer of antigen naïve (CD45RB^{hi}) CD4⁺ T cells into immunodeficient recipients led to colitis (3). The transferred T cells proliferated within the lymphopenic host, inducing effector functions that could be prevented by cotransfer of memory phenotype (CD4⁺CD45RB^{low}) T cells (3) and, more specifically, FoxP3⁺ CD4⁺ T regulatory (Treg) cells (4). Importantly, transfer of naïve CD4⁺ T cells into germ free mice

does not induce colitis indicating that inflammation in the gut results from a dysregulated immune response toward commensal microbes (5).

The gastrointestinal tract (GIT) contains immunological inductive sites, such as the Peyer's patches (PP), organized lymphoid aggregates and the adjacent mesenteric lymph nodes (MLN). The immune cells at these sites coordinate the balance between tolerance to food antigens and commensal microbes, whilst ensuring effective immune responses to mucosal pathogens. In humans, the chronic inflammatory bowel diseases (IBD), which include Crohn's disease (CD) and ulcerative colitis (UC), affect approximately 0.3% of the Western population (6) with increasing incidence worldwide (7). Antibody-neutralization studies have implicated cytokines (including tumor necrosis factor alpha (TNF α) and IL-12 p40) in the pathogenesis of CD, while the effectiveness of T-cell-ablative therapies have implicated T cells in UC (8). Bacteria reactive T lymphocytes have been more frequently observed in patients with IBD than in healthy individuals (9), leading to the proposal that a breakdown of tolerance toward the intestinal microflora plays a role in the pathogenesis of IBD.

The Gastrointestinal Tract and Chronic Inflammation

One pertinent question to the study of autoimmune and chronic inflammatory diseases is how inflammation in the GIT can influence the development of inflammation in distal tissues. Studies on a diverse array of inflammatory diseases indicate that the role of microbiota and the GIT barrier may extend well-beyond the gut (10). Accessory organs of the digestive system, connected to the small intestine by excretory ducts, include the pancreas and salivary glands, which are targets of the autoimmune diseases Type-1 diabetes (T1D) and Sjögren's Syndrome, respectively. The autoimmune disease that develops in non-obese diabetic (NOD) mice targets the pancreas and other accessory organs of the digestive system, namely, the salivary glands (11) and gallbladder (12). Antigen specificity is considered a prerequisite for the accumulation of T cells in the islet lesion (13), but the site of priming of diabetogenic T cells remains unknown. Lymphocytes infiltrating the pancreatic islets in both human T1D and NOD mice express $\alpha 4\beta 7$ -integrin, supporting a link between T1D and the gastrointestinal immune system (14). Furthermore, antibodies blocking either $\alpha 4\beta 7$ -integrin or its ligand, the mucosal addressin cell adhesion molecule (MadCAM-1), prevent diabetes in NOD mice (15, 16).

T Helper Cells in the GIT and Associated Lymphoid Tissues

In the GIT, T-dependent antibody responses are strongly biased toward IgA, which has a crucial role modulating immune responses to commensal microbiota and neutralizing intestinal pathogens (17, 18). IL-17 producing CD4⁺ (Th17) cells that express the transcription factor ROR γ t and the IL-23 receptor are enriched in normal intestines. Th17 cells contribute to intestinal homeostasis by regulating IgA secretion and play a critical biological function in clearing extracellular pathogens through

the release of effector cytokines (interleukin (IL); IL-17A, IL-17F, IL-21, and IL-22) (19). In this manner, Th17 cells also contribute to the pathology of inflammatory diseases, including IBD (20, 21). The production of affinity-matured antibody requires the interaction of B cells with a specialized subset of Th cells named T follicular helper (Tfh) cells (22), but the identity of Tfh cells that provide help to B cells in the GIT is only beginning to be understood.

CCR9⁺ T Helper Cells

Retinoic acid can induce the expression of integrin $\alpha 4\beta 7$ and the G protein coupled chemokine receptor 9 (CCR9) on lymphocytes, which allows their migration toward the GIT that expresses the chemokine ligand CCL25 (23). In healthy humans and mice, CCR9 is expressed predominately by a subset of T cells that migrate selectively to the gut (24, 25). Increased numbers of CCR9 expressing T cells have been observed in peripheral blood of patients with IBD (26). More recently, we described a T helper (Th) cell subset based upon expression of CCR9 (termed Tccr9 cells) that contribute to the regional specification of organ-specific autoimmune disease (27). Tccr9 cells constituted only a small fraction of CD4⁺ T cells in the lymphoid tissues and circulation of healthy mice and humans, but exhibited an inappropriate accumulation in the autoimmune lesions of the pancreas and salivary glands of NOD mice and were abundant in the peripheral blood of most Sjögren's syndrome patients. Tccr9 cells exhibited characteristics of T follicular helper (Tfh) cells-including expression of Bcl6, IL-21, c-Maf, and ICOS (27), suggesting that Tccr9 cells may be selectively recruited from a CCR9⁺ precursor population in the follicular environment of gut-associated lymphoid tissue.

In healthy humans, CCR9 is found primarily on T cells that selectively migrate to the GIT and is thought to play a role in several inflammatory disorders of the GIT. However, our studies demonstrate that during autoimmunity and chronic inflammation, CCR9⁺ T helper cells also infiltrate the pancreas and other accessory organs of the digestive system and are crucial to the destruction of these tissues. The implication of these findings is that T cells that are activated in the gut can disseminate to other organs to cause tissue damage. Here, we analyse CCR9⁺ Th and Tfh cells within the GIT and GIT associated lymphoid tissues to determine whether CCR9 expression and the characteristics of these populations reflect the state of inflammation.

RESULTS

GIT Inflammation in IL2^{-/-} Mice

Our previous studies demonstrated that the GIT-homing chemokine receptor CCR9 marked a subset of IL-21-producing Th cells in the inflamed lesions of the pancreas and salivary glands of T1D prone NOD mice (27). Examination of the phenotype of this population suggested a close relationship between CCR9⁺ Th cells and Tfh cells and we hypothesized that CCR9⁺ Th cells may emerge from Tfh-like cells in GIT lymphoid tissue. However, we had yet to analyse the characteristics of CCR9⁺ cells in the GIT and whether CCR9⁺ Th cells were

distinct under conditions of GIT inflammation. Therefore, we examined CCR9^{+/−} Th and CCR9^{+/−} Tfh cells in two models of autoimmunity and inflammation, namely the NOD mouse and mice that have been made genetically deficient in IL-2 (*Il2*^{−/−} mice). NOD mice exhibit a mild subclinical colitis (28), whereas *Il2*^{−/−} mice that lack IL-2 dependent Tregs and exhibit multi-organ autoimmunity, exhibit a chronically inflamed GIT (29). The GIT inflammation in *Il2*^{−/−} mice is influenced by microbiota as colitis is significantly reduced under germ-free conditions (30).

Histological analyses of the intestine of *Il2*^{−/−} and WT mice stained with hematoxylin and eosin showed increased leukocyte infiltration of the intestine of *Il2*^{−/−} mice (Figures 1A,B) compared with WT mice (Figures 1C,D). There were greater numbers of leukocytes (index of inflammation) in the GIT of *Il2*^{−/−} mice; increased numbers of CD45⁺ leukocytes in the spleen, mesenteric lymph nodes (MLN) (Figure 1E) and increased numbers of lamina propria lymphocytes (LPL) in the small intestine (SI LPL) compared with WT mice as shown by FACS analysis (Figure 1F). In the large intestine, there was a trend of increased numbers of LPL and intraepithelial lymphocytes (IEL) in *Il2*^{−/−} mice relative to WT mice (Figure 1F). By contrast, there were no differences observed in the numbers of CD45⁺ leukocytes in the Peyer's Patches (PP) or small intestine intraepithelial lymphocytes (SI IEL) (Figure 1F).

Increased Numbers of CCR9⁺ Th and Tfh Cells in the Inflamed GIT

We next examined the numbers of CD44^{hi} (activated/memory phenotype) CD4⁺ T cells expressing CCR9 compared with those that lack CCR9 expression across GIT and GIT-associated lymphoid tissues. The results demonstrate greater numbers of CCR9⁺ CD44^{hi} T cells in the inflamed GIT of *Il2*^{−/−} mice compared with WT mice in the spleen, MLN, SI IEL, SI LPL, but not in PP or LI IEL or LI LPL where the low cell numbers retrieved at these sites influenced group numbers (Figure 2A). When analyzed as a percentage of CD4⁺ CD44^{hi} T cells, CCR9⁺ cells were increased in the spleen and MLN of *Il2*^{−/−} mice relative to WT mice (Figure 2B). However, there were no statistical differences in the percentages of CCR9⁺ cells within the CD4⁺CD44^{hi} populations in the PP, SI IEL, LI IEL, LI LPL (Figure 2B). Taken together, these results show that *Il2*^{−/−} mice exhibit an expansion of CCR9⁺ Th cells, and there is a specific increase in the proportion of gut homing CCR9⁺ memory phenotype CD4⁺ T cells in the spleen, MLN and SI LPL.

Foxp3⁺ T follicular regulatory T (Tfr) cells are a specialized subset of Tregs cells that colocalize within B cell follicles of secondary lymphoid tissues and exhibit characteristics attributed to both Treg and Tfh cells, and are included within the C-X-C chemokine receptor 5 (CXCR5)⁺, programmed cell death protein (PD1)⁺ population of CD4⁺ T cells (29, 31, 32). To determine the percentages of Tfh cells in *Il2*^{−/−} and WT mice, we gated out Foxp3⁺ cells as *Il2*^{−/−} mice have a deficiency of IL-2 dependent FoxP3⁺ Treg cells (Figure 2C). The percentages of PD1⁺ CXCR5⁺ Foxp3[−] Tfh cells were significantly increased in both the MLN and PP of *Il2*^{−/−} mice compared with WT mice

(Figure 2D), as we have observed previously for ICOS⁺ CXCR5⁺ CD4⁺ T cells in *Il2*^{−/−} mice (29). Within the FoxP3[−] Tfh population, CCR9⁺ Tfh cells were increased in the PP of *Il2*^{−/−} mice compared with Tfh cells that lacked CCR9 (Figure 2D).

CCR9⁺ Th Cells in the GIT Exhibit Both Th17 and Th1 Characteristics

For the initial characterization of CCR9⁺ Th cells, we analyzed the expression of the surface markers C-C chemokine receptor type 6 (CCR6), the integrin α4β7, IL-9R, the IL-2 receptor beta chain (CD122), the IL-7 receptor (CD127), CXCR5, and PD-1 on the CCR9⁺ Th population by FACs (Figure 3). There was a trend of increased expression of the chemokine receptor CCR6 on CCR9⁺ Th cells in all lymphoid organs examined in both *Il2*^{−/−} and WT mice, which reached significance in the MLN (Figures 3A,B). Similarly, α4β7 (Figures 3C,D) and IL-9R (Figures 3E,F) were consistently coexpressed with CCR9 on CD4⁺ T cells in the MLN and PP in both *Il2*^{−/−} and WT mice. The percentage of CD4⁺ T cells expressing IL-2R beta (CD122), was significantly increased on CCR9⁺ CD4⁺ T cells within the SI IEL of the *Il2*^{−/−} mice (Figures 3G,H). In contrast to *Il2*^{−/−} mice, WT mice harbored an increased percentage of CCR9⁺ CD4⁺ T cells expressing IL-7R (CD127) in the SI LPL (Figures 3I,J). Whilst the overall percentages of CCR9⁺ Tfh cells were increased in *Il2*^{−/−} mice compared with WT mice (Figure 2D), the percentages of CCR9⁺ Th cells co-expressing CXCR5 were increased in the PP of WT mice (Figures 3K,L). By contrast, the percentages of CCR9⁺ Th cells co-expressing PD-1 were increased in the MLN of *Il2*^{−/−} mice (Figures 3M,N).

To further analyze the function of CCR9 expressing cells in the chronically inflamed GIT of *Il2*^{−/−} mice relative to IL-2 sufficient WT mice that do not develop GIT inflammation, we determined the expression of the pro-inflammatory cytokines (IL-17, IL-22, IL-21, TNFα, IFNγ) and the anti-inflammatory cytokines (IL-10, IL-4), which may be relevant to the chronic inflammation observed in *Il2*^{−/−} mice (29). One of the most striking features of CCR9⁺ Th cells in the GIT was the increased fraction of IL-17 producing cells. We observed a greater percentage of CD4⁺ T cells expressing IL-17 in *Il2*^{−/−} compared with WT mice, and a greater percentage of IL-17 producing Th cells were CCR9⁺ (Figures 4A,B). These findings indicate that IL-17 is commonly coexpressed with CCR9 on CD44^{hi} CD4⁺ T cells in the GIT. IL-22 also produced by Th17 cells, with both pro-inflammatory and regenerative functions. CD4⁺ T cells from *Il2*^{−/−} mice harbored a greater fraction of IL-22 producing cells than WT mice, but this was only significantly increased within the population of Th cells that lacked CCR9 expression (Figures 4C,D). The cytokine IL-21 is produced by both Tfh cells (33) and Th17 (34) cells, and CCR9⁺ IL-21-producing cells were also increased in both the MLN and PP of *Il2*^{−/−} mice relative to WT mice (Figures 4E,F), providing a source of IL-21 that may explain our previous observation of increased amounts of IL-21 in *Il2*^{−/−} mice (29).

Tumor necrosis factor (TNFα) is thought to contribute to the pathology of IBD in humans and mice (35). The percentages of TNFα-producing CCR9⁺ Th cells were significantly increased in the PP of *Il2*^{−/−} mice compared with WT mice (Figures 4G,H).

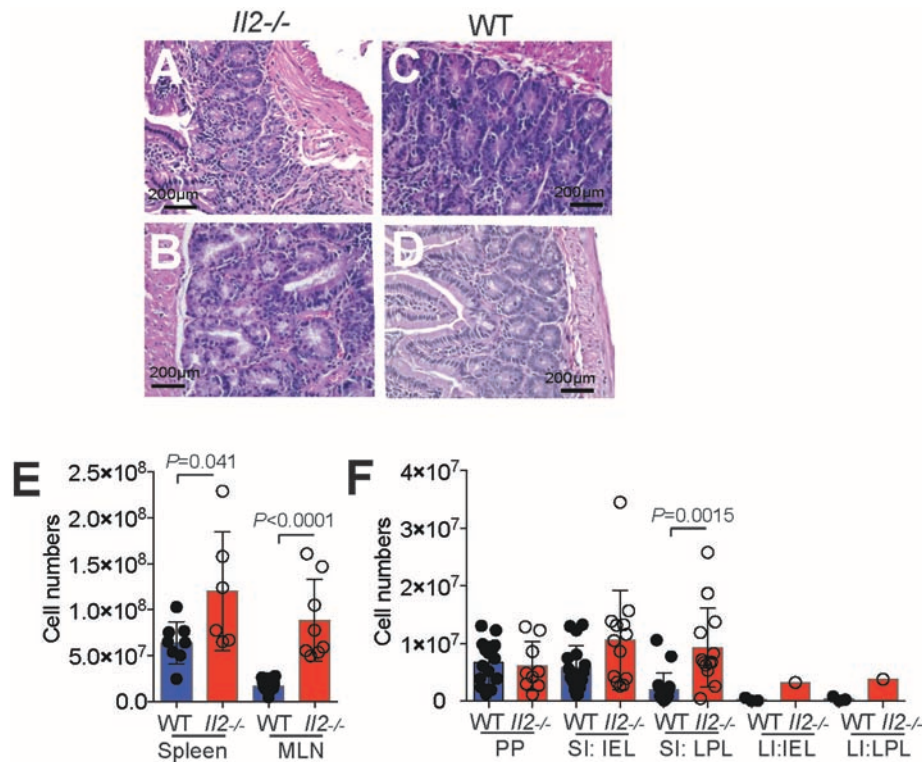


FIGURE 1 | Inflamed bowel and numeration of leukocytes in *Il2*^{-/-} mice. Representative histological sections of intestinal tissues from *Il2*^{-/-} mice (A,B) and WT mice (C,D) mice stained with hematoxylin and eosin, optical microscope 40X. (E,F) FACS analyses and cell counts were used to determine numbers of CD45⁺ cells in *Il2*^{-/-} and WT mice, cell numbers were normalized to organ weight. Data shown as mean ± SEM (*n* = 3-9) and analyzed by Students *T*-test.

An increased percentage of TNF α producing SI IEL was also observed in *Il2*^{-/-} mice, and the percentages of TNF α -producing SI LPL expressing CCR9 or lacking CCR9 were greater in the *Il2*^{-/-} mice (Figure 4D). Taken together, these findings indicate that there was not an associated coexpression with CCR9, but TNF α -expressing CD4⁺ T cells were related to the deficiency of IL-2 and to the chronic inflammation in these mice. By contrast, the percentages of IFN γ producing Th cells in *Il2*^{-/-} and WT mice were largely similar (Figures 4I,J). However, there was a significantly increased fraction of IFN γ + CCR9⁻ CD4⁺ cells in the MLN in *Il2*^{-/-} mice relative to the MLN of WT mice (Figures 4I,J). We also investigated the percentages of CD4⁺ T cells expressing the anti-inflammatory interleukins, IL-4 and IL-10. There was a marked trend of increased percentages of IL-10 expressing cells in the PP and MLN of WT mice compared to the *Il2*^{-/-} mice (Figures 4K,L). For IL-4, the fraction of CD4⁺ T cells expressing IL-4 was not significantly different between groups (Figures 4M,N).

Tfh cells have been reported to acquire effector functions associated with other T helper subsets, producing IL-17 (36, 37), IL-4 (38), and IFN γ in some studies, but not IL-17 (33, 39) or IL-4 (38) in others. We analyzed both CCR9⁺ and CCR9⁻ Tfh cells in the PP and MLN for the production of the cytokines IL-17 and IL-21 by intracellular immunostaining and FACS analyses. *Il2*^{-/-} mice contained the greatest percentages of IL-21 producing cells

within the CCR9⁺ population in both the PP and MLN relative to CCR9⁻ Tfh cells (Figure 5B). Whereas, CCR9⁺ Tfh cells from WT mice contained more IL-21-producing cells in the MLN, but not the PP (Figures 5A,B). Intracellular detection of IL-17 demonstrated that CCR9⁺ Tfh cells from the PP of *Il2*^{-/-}, but not WT, mice contained a greater percentage of IL-17 producing cells (Figure 5C). By contrast, the CCR9⁺ Tfh population also contained a greater percentage of IL-17 producing cells than the CCR9⁻ Tfh population in the MLN of both WT and *Il2*^{-/-} mice (Figure 5D).

CCR9⁺ Th and Tfh Cells Exhibit a Site-Specific Transcriptome

Analyses of *Il2*^{-/-} mice indicated that CCR9⁺ Th cells and CCR9⁺ Tfh cells are phenotypically distinct from their CCR9⁻ counterparts. As discussed earlier, type-1 diabetes prone NOD mice harbor an increased number of IL-21-producing CCR9⁺ Th cells in the inflamed lesions of the pancreas and salivary glands that are phenotypically similar to Tfh cells (27). As CCR9 is a GIT-homing chemokine receptor, we questioned whether CCR9⁺ Th cells in the pancreas derive from CCR9⁺ Tfh cells in the GIT. Therefore, we determined the phenotypic relationship between CCR9⁺ Th and Tfh cells in the GIT and pancreas of NOD mice by differential gene expression analyses. RNA was

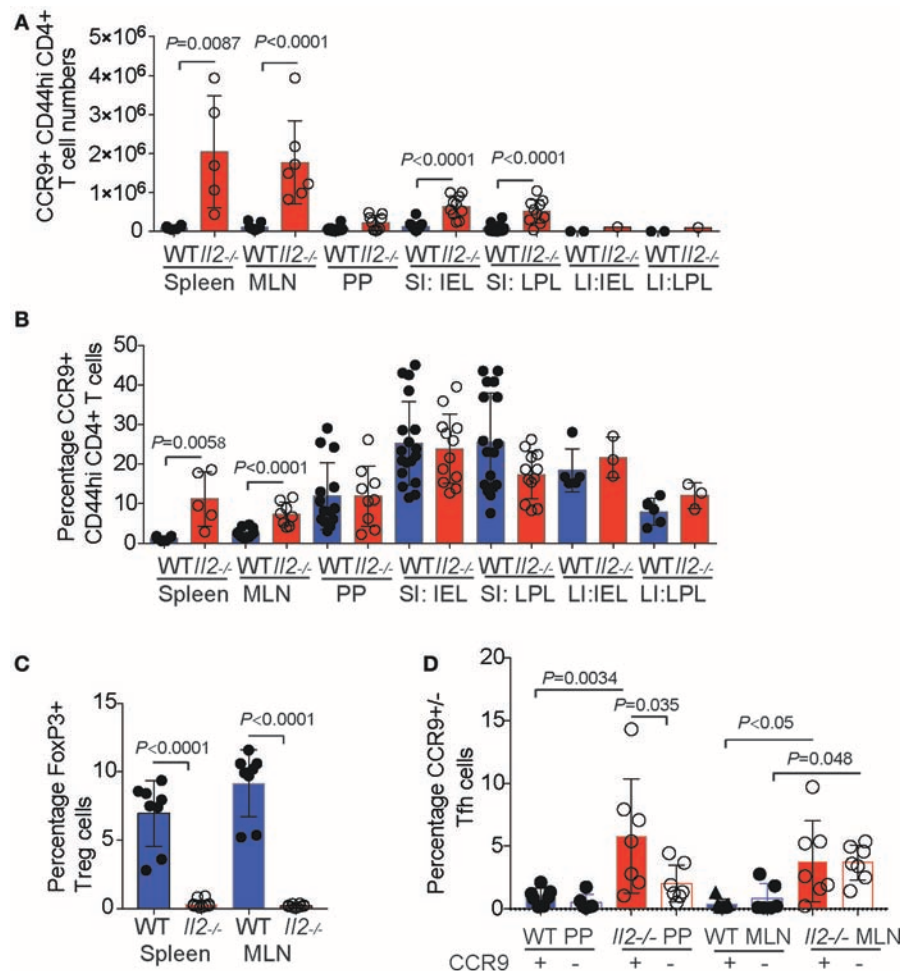


FIGURE 2 | Expansion of CCR9⁺ CD4⁺ T cells in Il2^{-/-} mice. Numeration **(A)** and percentages **(B)** of CCR9⁺ and CCR9⁻ CD44^{hi} CD4⁺ T cells in Il2^{-/-} and WT mice determined by FACS analysis. (MLN) mesenteric lymph nodes; (PP) Peyer's Patches; (SI IEL) intraepithelial lymphocytes of the small intestine; (SI LPL) lamina propria lymphocytes of the small intestine; (LI IEL) intraepithelial lymphocytes of the large intestine; (LI LPL) lamina propria lymphocytes. **(C)** Percentages of FoxP3⁺ CD4⁺ TCRβ⁺ T regulatory (Treg) cells in the spleen and MLN and **(D)** percentages of CXCR5⁺ PD1⁺ FoxP3⁻ CD4⁺ TCRβ⁺ T follicular helper (Tfh) cells in the PP and MLN of Il2^{-/-} and WT mice. Data shown as mean ± SD, where *n* = 3–15 female mice 7–9 weeks of age. Statistical significance was analyzed by students *T*-test.

extracted from FACs sorted CCR9⁺ and CCR9⁻, CXCR5⁺, PD-1⁺ TCRβ⁺, CD4⁺ Tfh cells from the PP and from CCR9⁺ and CCR9⁻, CXCR5⁻, TCRβ⁺, CD4⁺ cells from the pancreas. Gene expression was determined by SurePrint G3 Mouse GE 8x60K Microarray Kit from Agilent technologies and revealed a greater upregulation of genes in the CCR9⁺ populations. Therefore, we focused on the genes with increased expression in CCR9⁺ Th and Tfh cells in both tissues.

The PP Tfh cell populations exhibited a clear Th17 transcriptome relative to the pancreas Th populations, indicating a microenvironment-specific signature (**Figure 6A**). Genes upregulated in the PP compared with the pancreas included genes known to be expressed in Tfh cells (as would be expected by a comparison of Th and Tfh cells) *Cxcr5*, *Icos*, *Bcl6*, *Maf*, *Il21* and Th17 signature genes *Il17a*, *Il21*, *Il23r*, *Il17f*, *Il22* (**Figure 6A**). Th17 signature genes were more enriched in CCR9⁺ Tfh cells relative to CCR9⁻ Tfh cells within the PP (**Figure 6B**). These

data indicated that both CCR9⁺ and CCR9⁻ Tfh cells in the PP share characteristics of Th17 and Tfh genes, but also demonstrate notable differences; CCR9⁺ Tfh cells in the PP express increased amounts of *Ccr9*, *Il21*, *Il-22ra*, *v-Maf*, *Lifr*, *Cxcl13*, the cytokine and cytokine receptors *Il20*, *Il18bp*, *Il28ra* compared with CCR9⁻ Tfh cells in the PP (**Figure 6B**).

When we compared CCR9⁺ and CCR9⁻ Th cells in the pancreas, several of the most DE genes in CCR9⁺ cells at this site were amongst the most DE genes in CCR9⁺ Tfh cells in the PP. They included; *Ccr9*, *Cxcl9*, *Aif1*, *Rxrg*, *Cx3cr1*, *Lirb3*, and *Tnfrsf2* (**Figure 6C**). Pancreatic CCR9⁺ Th cells were also distinct from their CCR9⁻ counterparts in the pancreas by increased expression of genes known to be expressed by Tfh or Th17 cell, including *Il21*, *Il17rc*, *Fgfr1*, *Ccl6* (**Figure 6C**).

It was of interest to observe some clear similarities between the most differentially expressed genes in CCR9⁺ Th cells from the pancreas and in CCR9⁺ Tfh cells from the PP that suggested

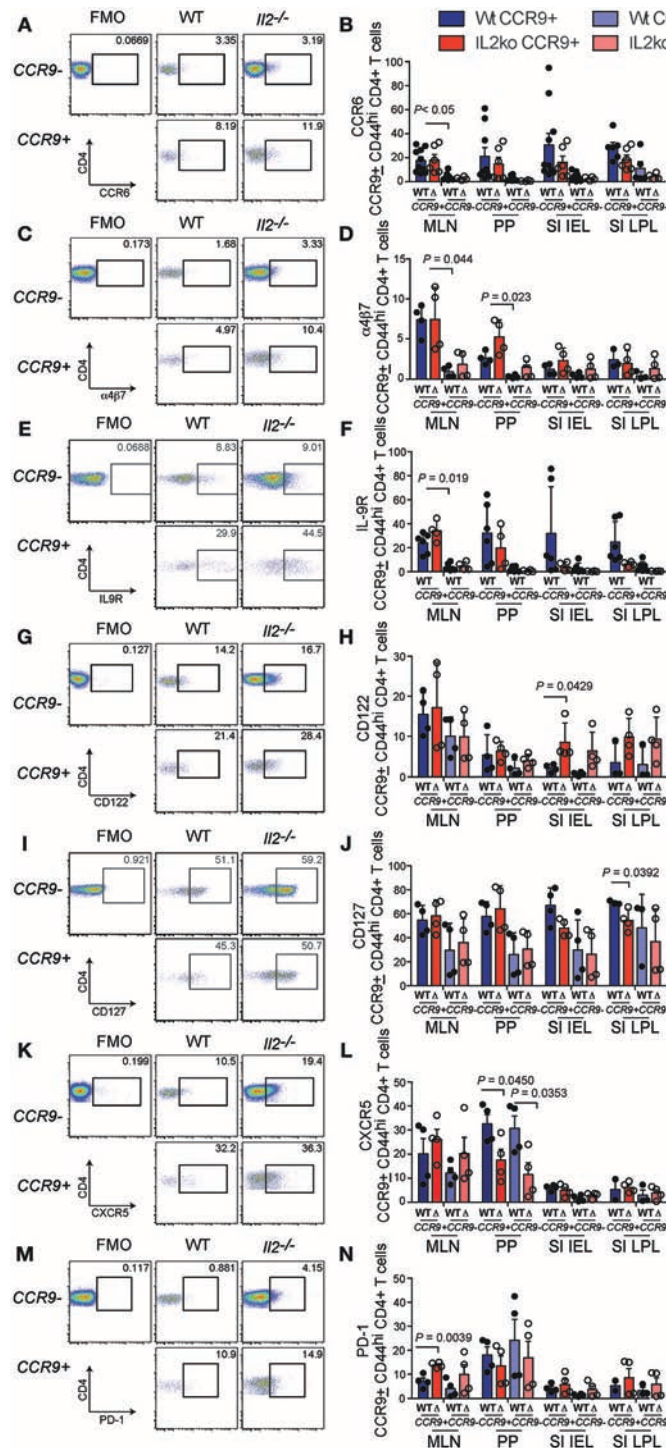


FIGURE 3 | Distinct phenotypic profile of CCR9⁺ Th cells in the inflamed GIT. Flow cytometric analyses showing representative FACS dot plots of surface marker expression for CCR9⁺ and CCR9⁻ CD44^{hi} CD4⁺ T cells from the mesenteric lymph nodes and quantitation of the percentages of CCR9⁺ and CCR9⁻ CD44^{hi} CD4⁺ T cells from *Il2*^{-/-} and WT mice. CCR6; (A) representative FACS dot plot and (B) quantitation. a4b7; (C) representative FACS dot plot and (D) quantitation. IL9R; (E) representative FACS dot plot and (F) quantitation. CD122; (G) representative FACS dot plot and (H) quantitation. CD127; (I) representative FACS dot plot and (J) quantitation. CXCR5; (K) representative FACS dot plot and (L) quantitation. PD1; (M) representative FACS dot plot and (N) quantitation. (MLN) mesenteric lymph nodes; (PP) Peyer's Patches; (SI IEL) intraepithelial lymphocytes of the small intestine; (SI LPL) lamina propria lymphocytes of the small intestine; (LI IEL) intraepithelial lymphocytes of the large intestine; (LI LPL) lamina propria lymphocytes of the large intestine. Data shown as mean + SD, where n=3-9 female mice at 7-9 weeks of age. Statistical significance was assessed by students T-test.

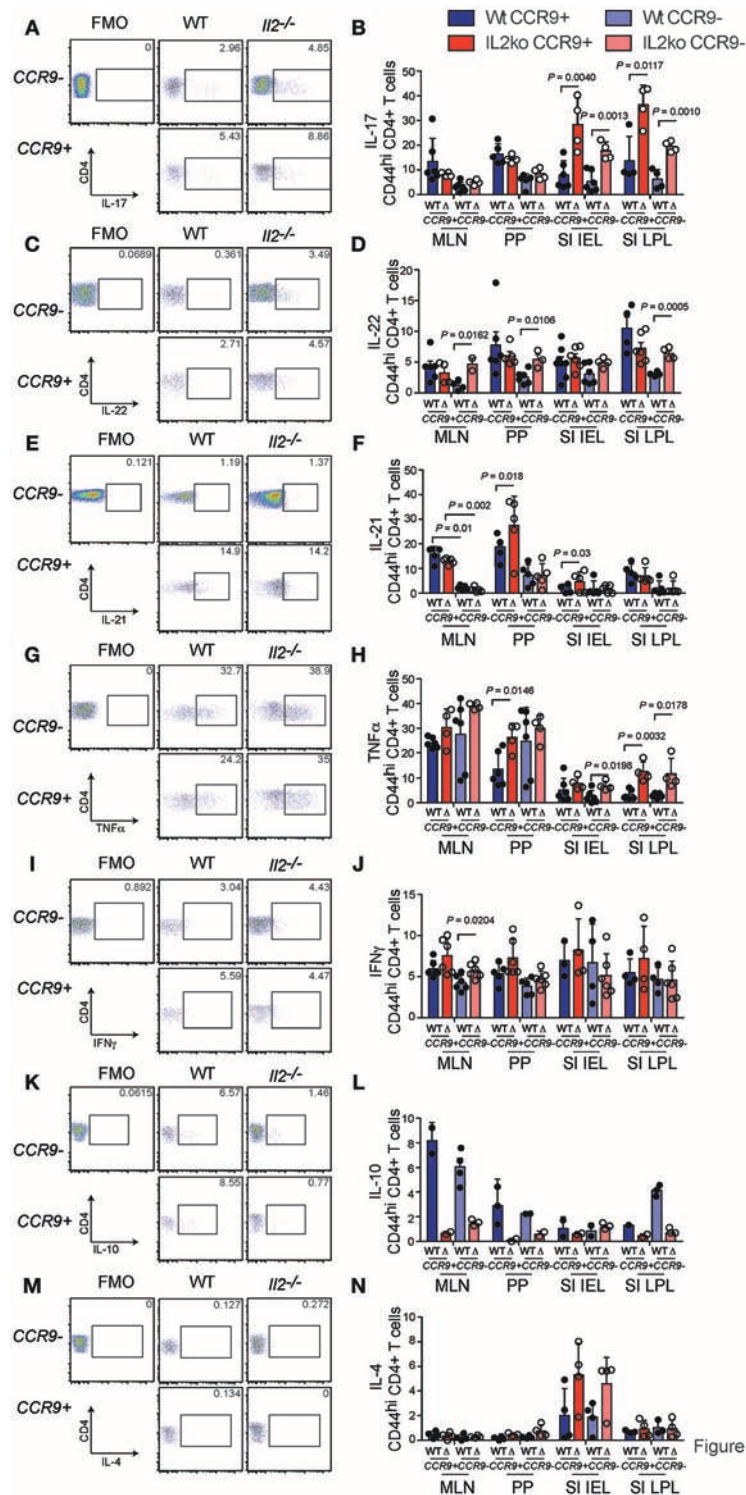
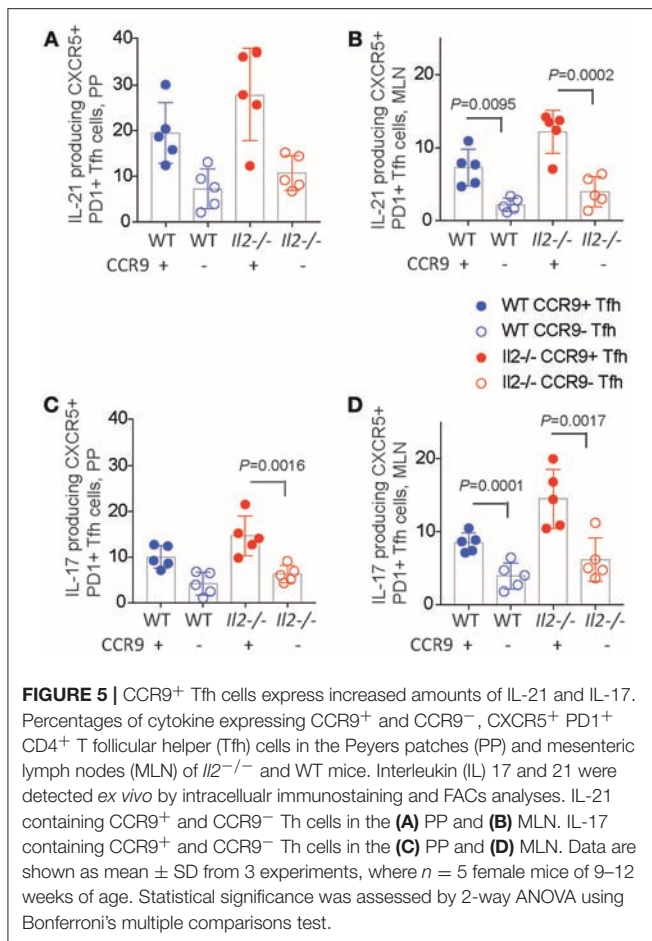


FIGURE 4 | CCR9⁺ CD4⁺ Th cells exhibit a distinct cytokine profile in the inflamed GIT. Detection of cytokines in CCR9⁺ and CCR9⁻ Th cells from GIT associated lymphoid tissues of *Il2*^{-/-} and WT mice by intracellular immunostaining and FACS analyses. **(A)** Representative FACS dot plot and **(B)** quantitation of IL-17. **(C)** Representative FACS dot plot and **(D)** quantitation of IL-22. **(E)** Representative FACS dot plot and **(F)** quantitation of IL-21. **(G)** Representative FACS dot plot and **(H)** quantitation of TNFα. **(I)** Representative FACS dot plot and **(J)** quantitation of IFNγ. **(K)** Representative FACS dot plot and quantitation **(L)** of IL-10. **(M)** Representative FACS dot plot and **(N)** quantitation of IL-4. MLN, mesenteric lymph nodes; PP, Peyer's Patches; SI IEL, intraepithelial lymphocytes of the small intestine; SI LPL, lamina propria lymphocytes of the small intestine; LI IEL, intraepithelial lymphocytes of the large intestine; LI LPL, lamina propria lymphocytes of the large intestine. Data are shown as mean ± SD, where *n* = 3–6 female mice of 7–9 weeks of age. Statistical significance was analyzed by students *T*-test.



a GIT microenvironment, such as the retinoid receptor *Rxrg*, and Th17 cells or IL-17 interactions (Figures 6B,C). Taken together, these findings indicate that CCR9⁺ Th cells in the inflamed lesions of the pancreas in NOD mice are phenotypically related to GIT-residing CCR9⁺ Tfh cells, supporting the notion that IL-21-producing CCR9⁺ Th cells that are critical for the development of T1D may emerge from Tfh cells in the GIT or a common GIT-residing precursor population.

The results from our microarray indicated some variation between samples that may reflect differences in inflammation and the disease progression between individual female NOD mice. Therefore, to validate our findings we performed qPCR on selected genes in CCR9⁺ and CCR9⁻ populations from the PP and pancreas (Figure 7). *Il21*, *Cxcl13*, *Rxrg*, *Il17a*. The expression of genes by qPCR was normalized based on the level of the housekeeping gene *Rpl19* and shown as fold modulation of CCR9⁺ Th and Tfh cells relative to CCR9⁻ Th and Tfh cells, respectively. *Il21* was increased in both CCR9⁺ Th and Tfh cells compared with CCR9⁻ cells, where it was most highly expressed in the CCR9⁺ Tfh population (Figure 6D). *Tnfrsf2*, *Rxrg* and *Cxcl13* were also significantly increased in both CCR9⁺ Th and CCR9⁺ Tfh cells compared with their CCR9⁻ counterparts (Figure 6D). By contrast, expression of *Il9r* was not increased in either CCR9⁺ populations compared with CCR9⁻ cells

(Figure 6D). Taken together, these data support our microarray findings and show that CCR9⁺ distinguishes both Th and Tfh cell populations.

CCR9⁺ Th and Tfh Cells Exhibit a Site-Specific Phenotype

Flow cytometric analyses of CCR9⁺ and CCR9⁻ Th and Tfh cell populations in the lymphoid tissues of the GIT, pancreas and pancreatic lymph nodes in NOD mice revealed that an increased percentage of CCR9⁺ cells in NOD mice express an array of cytokines in the GIT relative to CCR9⁻ cells. CCR9⁺ Th cells from NOD mice contained a greater fraction of IL-21 producing cells in the spleen, MLN, PLN and pancreas compared with CCR9⁻ Th cells (Figure 7A). IL-17 producing cells were concentrated in the CCR9⁺ populations in the spleen, MLN and PP (Figure 7B). CD4⁺ Th cells from NOD mice exhibited a greater fraction of IL-22 producing cells than WT mice in all tissues, significantly increased within the MLN, PP and LPL populations (Figure 7C). CCR9⁺ Th cells in the MLN, PP, and SI LPL contained the greatest percentages of IL-22 producing cells (Figure 7C). In addition, there was an increased fraction of IFN γ -producing CCR9⁺ Th cells in the spleen, PP and SI LPL (Figure 7D). By contrast, CCR9⁻ Th cells contained a greater fraction of IFN γ producing cells in the pancreas compared with CCR9⁺ Th cells (Figure 7D). TNF α -producing cells, in turn, were enriched in the CCR9⁺ Th fraction in the MLN alone of NOD mice (Figure 7E).

Similar to the cytokine profile of CCR9⁺ Th cells; CCR9 expression distinguished the Tfh population with the highest fraction of both IL-17 (Figure 7F) and IL-21 (Figure 7G). Taken together, these findings are consistent with our microarray findings and suggest that CCR9-expressing Th and Tfh cells contain differentiated/effector cells that express an array of cytokines, notably cytokines that are associated with Th17-like cells and differentiated Th cells within the GIT microenvironment.

CCR9⁺ Tfh and CCR9⁺ Th Cells From GIT Lymphoid Tissues Migrate to the Pancreas

Previous analyses of CCR9⁺ Th cells from the inflamed pancreas of pre-diabetic NOD mice indicated some phenotypic similarities with Tfh cells and we hypothesized that CCR9⁺ Th cells may emerge from Tfh-like cells in GIT lymphoid tissue (27). To test whether Tfh cells from GIT-associated lymphoid tissue could migrate to the pancreas, we performed an adoptive transfer experiment using FACS sorted CFSE labeled cell subsets. CCR9⁺ Tfh cells, CCR9⁻ Tfh cells, CCR9⁺ Th cells, and CCR9⁻ Th cells were sorted from the PP and MLN of 12–14 week old female NOD mice (Figure 8A), CFSE labeled and then transferred into 12 week old (pre-diabetic) NOD female recipients. Four days later, CFSE⁺ cells were recovered from the PP, MLN and pancreas and analyzed by immunostaining for CXCR5 and CCR9 by FACS. The results show that CCR9⁺ Th cells from the GIT lymphoid tissues migrated to and accumulated in small numbers in the pancreas (Figure 8B) the MLN (Figure 8C) and the PP

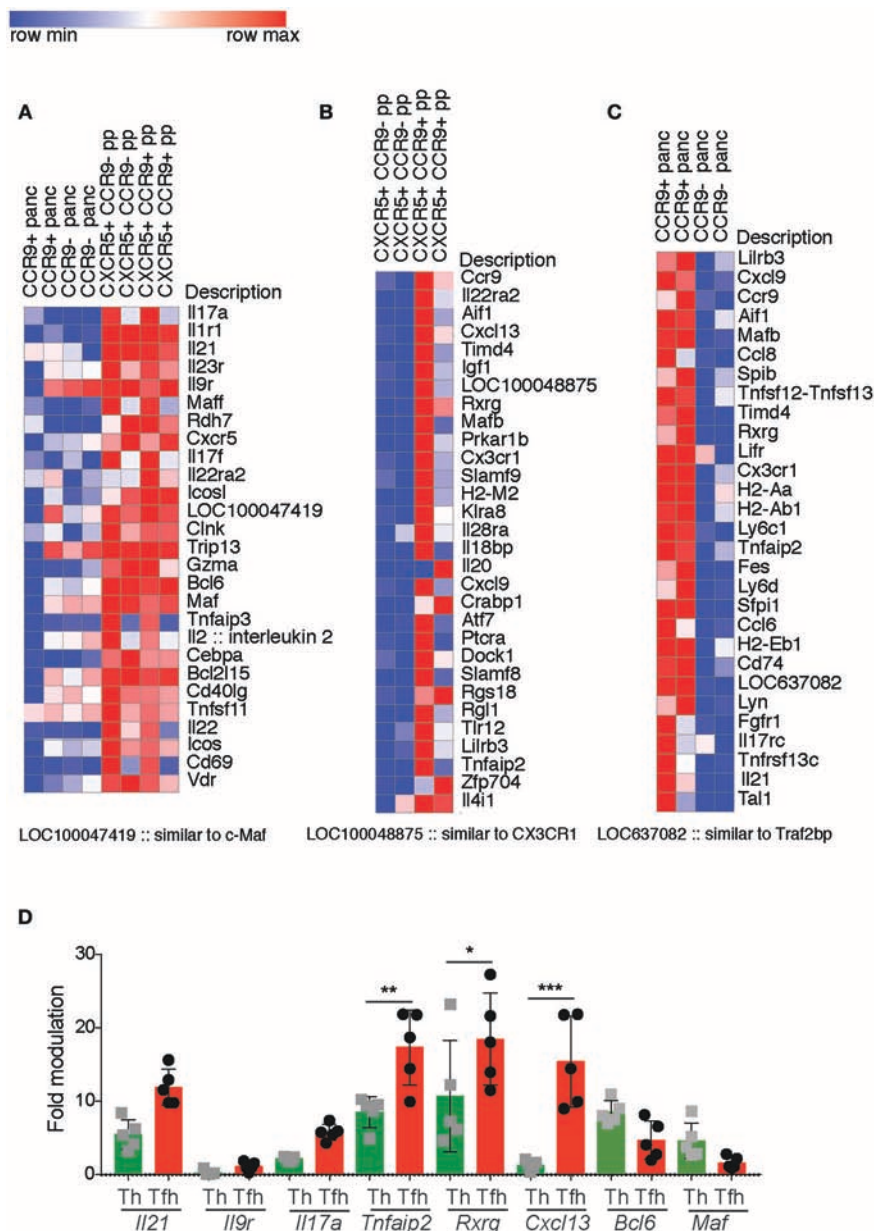


FIGURE 6 | Differentially expressed genes in CCR9⁺ relative to CCR9⁻ Tfh from the Peyer's patches and CCR9⁺ relative to CCR9⁻ Tfh cells from the pancreas infiltrate from non-obese diabetic (NOD) mice. Gene expression was determined by SurePrint G3 Mouse GE 8x60K Microarray Kit from Agilent technologies. Genes selected from the 50 most differentially expressed (DE) genes shown in heat maps, Log2 Fold difference of 2.5–5.3 (fold difference of 5.6–34.6). **(A)** relatively higher expression of Th17 signature genes in CCR9⁺ T follicular helper (Tfh) cells from the Peyer's patches compared with CCR9⁺ T helper (Th) cells from the pancreas of 10–12 week old female NOD mice. **(B)** DE genes from Peyer's patch CCR9⁺ Tfh cells relative to Peyer's patch CCR9⁻ Tfh cells. **(C)** DE genes from CCR9⁺ Tfh cells relative to CCR9⁻ Tfh cells from the pancreas infiltrate of 12 week old female NOD mice. **(D)** qPCR validation of DE genes selected from **(A–C)**. Gene expression of *Il21*, *Il9r*, *Il17a*, *Tnfrsf11*, *Rxrg*, and *Cxcl13* from CCR9⁺ Tfh or Th cells analyzed by real-time PCR relative to *Rpl19* expression. Data are shown as fold modulation of gene expression in CCR9⁺ Tfh relative to CCR9⁻ Tfh cells or CCR9⁺ Tfh cells relative to CCR9⁻ Tfh cells, where *n* = 5 mice per group. Statistical significance was assessed by 2-way ANOVA using Bonferroni's multiple comparisons test. **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

(Figure 8D). By contrast, Th cells that lacked expression of CCR9 migrated to or accumulated poorly at all three sites.

The expression of CCR9 was similarly important for Tfh cells from the PP and MLN that had migrated into the pancreas, which were predominantly those that had lost expression of

CXCR5, but had retained CCR9 (Figure 8B). It is unlikely that the Tfh cells had lost CFSE due to proliferation as we have observed few endogenous Tfh cells in the pancreas (27). Previous studies have shown that Tfh cells maintain plasticity and can downregulate CXCR5 (40) and those data are consistent with our

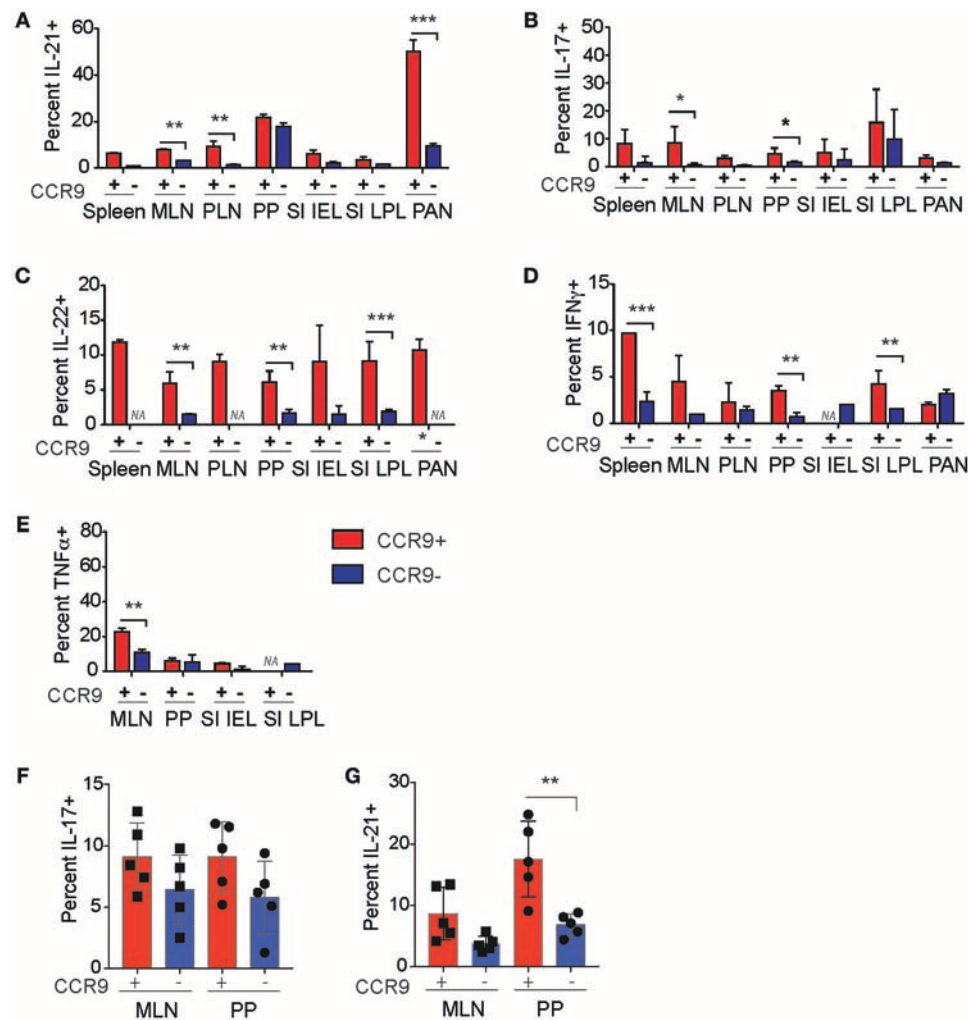


FIGURE 7 | CCR9 marks populations of Th and Tfh cells with enhanced cytokine expression in autoimmune inflammation. Percentages of cytokine expressing CCR9⁺ and CCR9⁻ CD4⁺ T helper (Th) and T follicular helper (Tfh) cells in the GIT and inflamed pancreas of NOD mice. CCR9⁺ and CCR9⁻ Th cells that contain (A) IL-21, (B) IL-17, (C) IL-22, (D) IFN γ , and (E) TNF α in the spleen. MLN, mesenteric lymph nodes; PP Peyer's Patches; SI IEL, intraepithelial lymphocytes of the small intestine; SI LPL, lamina propria lymphocytes of the small intestine; LI IEL, intraepithelial lymphocytes of the large intestine; LI LPL, lamina propria lymphocytes of NOD mice. Percentages of (F) IL-17 and (G) IL-21 expressing CCR9⁺ and CCR9⁻ Tfh cells in the mesenteric lymph nodes (MLN) and Peyer's patches (PP) of NOD mice. Cytokines were detected by intracellular immunostaining and FACs analyses. Data is shown as mean $\pm$ SD from 3 experiments, female mice 11–12 weeks of age $n = 5$ /group for Tfh cell analyses and $n = 7$ /group for Th cell analyses. Statistical significance was assessed by 2-way ANOVA using Bonferroni's multiple comparisons test. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

findings. By contrast, adoptively transferred CFSE⁺ Tfh cells that were retrieved from the PP and MLN contained a mixture of phenotypes. Four days after the CCR9⁺ Tfh cells transfers, we observed cells that expressed both CXCR5 and CCR9, as well as cells expressing reduced levels of both molecules (Figure 8A). CXCR5⁺ CCR9⁺ Tfh cells were retrieved in greater numbers from the MLN than CCR9⁻ (CXCR5⁺) Tfh cells or CCR9⁺ Th cells (Figure 8C). By contrast, whilst there was a trend of increased recovery of Tfh cells from the PP, there were no significant differences observed between the recovery of each of the 4 populations from the PP (Figure 8D). These findings demonstrate that Tfh cells from the GIT associated lymphoid tissues retain plasticity and that the expression of CCR9 is

important for the migration of GIT lymphoid tissue Tfh cells and Th cells from the bloodstream into the pancreas.

DISCUSSION

Previous examination of the phenotype of CCR9⁺ Th cells in the inflamed lesions of the pancreas of T1D-prone NOD mice suggested a close relationship between CCR9⁺ Th cells and Tfh cells and we hypothesized that CCR9⁺ Th cells may emerge from Tfh-like cells in GIT lymphoid tissue (27).

Using a combination of gene expression analyses and flow cytometric analyses of cells in the GIT and GIT associated

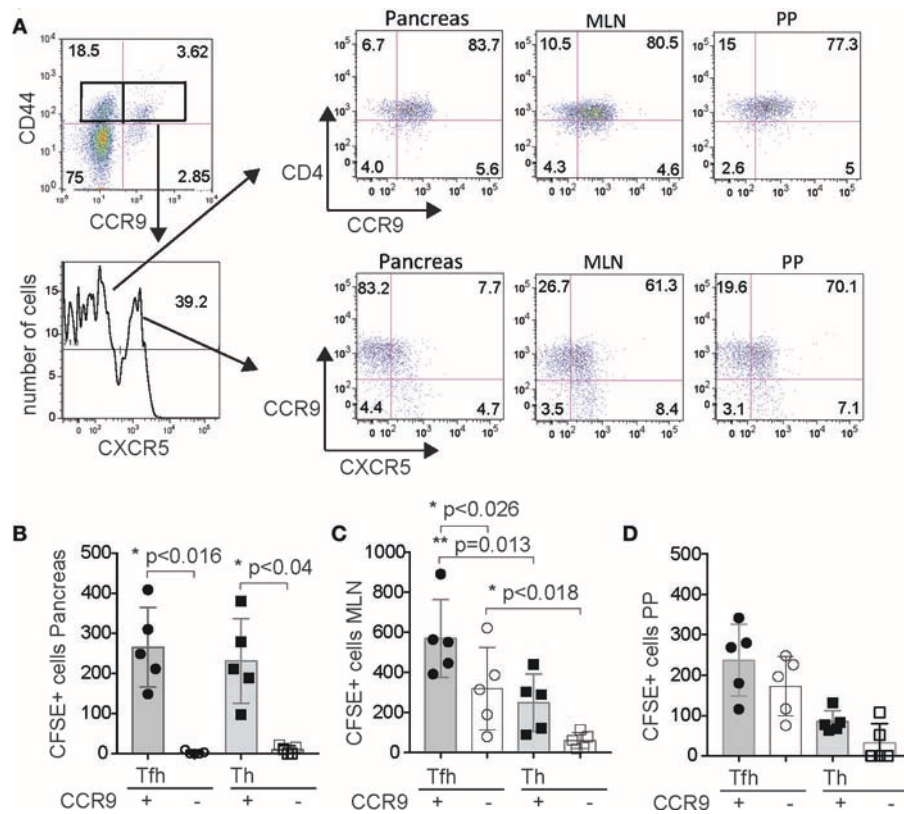


FIGURE 8 | CCR9 marks GIT-derived Tfh cells and Th cells that migrate to the pancreas. **(A)** FACS sorting strategy showing representative FACS dot plots gated on CD4 and then CD4 vs. CCR9 and FACS histogram of CXCR5 expression for purification of CCR9⁺ CXCR5⁺ Tfh cells, CCR9⁺ CXCR5⁺ Th cells, CCR9⁺ CXCR5⁻ Th cells and CCR9⁺ CXCR5⁻ Th cells from the PP and MLN, and representative FACS dot plots of CCR9⁺ Th cells and CCR9⁺ Tfh cells from the pancreas infiltrate, mesenteric lymph nodes (MLN) and Peyer's patches (PP). 6×10^5 CFSE labeled cells from each of the 4 subsets were injected (i.v.) into age matched female NOD recipients ($n = 5$ /transfer group). Quantitation showing numbers of CFSE labeled CD4⁺ CCR9⁺ and CD4⁺ CCR9⁻ Tfh and Th cells recovered from the **(B)** pancreas, **(C)** mesenteric lymph nodes and **(D)** Peyer's patches of NOD mice 4 days after adoptive transfer. Statistical significance was assessed by 2-way ANOVA using Bonferroni's multiple comparisons test.

lymphoid tissues, this study demonstrates a microenvironment specific signature for Tfh cells and Th cells that express the gut-homing chemokine receptor CCR9. We demonstrate that the expression of CCR9 marks the Tfh cells (and Th cells) that derive from the peyer's patches and mesenteric lymph nodes that can migrate into the inflamed lesions of the pancreas in NOD mice.

Several studies have demonstrated that lymphocytes infiltrating the islets in T1D humans and the NOD mouse express $\alpha 4\beta 7$ -integrin, supporting a link between T1D and the gastrointestinal immune system (41–44). In addition, antibodies blocking $\alpha 4\beta 7$ or MadCAM-1, prevent diabetes in NOD mice (15). The findings presented here reveal a closer association between the phenotype and transcriptome of Tfh cells that express the GIT homing receptor CCR9 and CCR9⁺ Th cells in the inflamed pancreas of NOD mice (27) than was previously appreciated. CCR9⁺ Tfh cells in the GIT and CCR9⁺ Th cells in the pancreas express common gene sets and express greater amounts of cytokines, such as IL-21 and IL-17, than their CCR9⁻ counterparts at the same sites. The production of IL-21 is a feature of both CCR9⁺ Th and CCR9⁺ Tfh cells, which is

critical for Th cells to provide help to both CD8⁺ T cells and B cells.

High levels of CCR9 have previously been detected in SI lymphocytes (45) and in the small bowel during IBD (46). Here, we analyzed the characteristics of Tfh cells and Th cells that express CCR9 in the GIT associated lymphoid tissues of *Il2*^{-/-} mice that exhibit chronic inflammation in the GIT. The expression of CCR9 marked a population of Tfh cells and Th cells with an increased capacity for cytokine production, which may suggest an increased level of activation or differentiation. CCR9⁺ Th cells in the inflamed GIT of *Il2*^{-/-} mice additionally exhibited increased expression of CCR6 and $\alpha 4\beta 7$ compared with their CCR9⁻ Th counterparts. Co-expression of the integrin $\alpha 4\beta 7$ and CCR9 further emphasizes the GIT-specific nature of CCR9⁺ Th cells as $\alpha 4\beta 7$ specifies the recruitment of T cells to the intestinal mucosa through its interaction with its ligand MadCAM-1, where it is critical in a TNF α -dependent model of CD (47). CCR6, in turn, is expressed on Th17 cells and has been associated with CD (48).

Co-expression analyses of CCR9 and cytokine production demonstrated an increased expression of cytokines that are typically produced by Th17 cells in both CCR9⁺ Th and Tfh cells in *Il2*^{-/-} mice compared to these populations in WT mice. Our findings indicate that IL-17 is commonly coexpressed with CCR9 on CD44^{hi} CD4⁺ T cells in the GIT, which contrasts with the lack of IL-17 production in CCR9⁺ Th cells purified from the inflamed pancreas of NOD mice (27). CCR9⁺ T cells express pro-inflammatory cytokines in both CD and UC (26, 49, 50). Whilst our findings are consistent with increased production of IL-17 from CCR9⁺ T cells isolated from CD LPL (50) we did not observe increased IFN γ as shown previously in CD in humans (50). TNF α was also produced by a greater fraction of PP and SI LPL CCR9⁺ Th cells in the inflamed GIT of *Il2*^{-/-} mice compared with WT mice, which is consistent with the inflammatory environment. Taken together, our findings demonstrate an increase in the production of pro-inflammatory cytokines, especially by CCR9⁺ Th and Tfh cells, in the inflamed GIT.

The expression of the GIT-homing chemokine receptor CCR9 distinguished populations in both the inflamed lesions of the pancreas and GIT associated lymphoid tissues. We have previously shown that NOD mice contain small numbers of IFN γ -producing CD4⁺ T cells in the pancreatic islet lesion, but they were largely restricted to the CCR9⁻ population (27). By contrast, CCR9⁺ Th cells from the GIT associated lymphoid tissues of NOD mice produced more cytokines than CCR9⁻ Th cells, including the Th1 cytokines TNF α and IFN γ , and the Th17 cytokines IL-17, IL-21, and IL-22. The microenvironment of the GIT favored a Th17-like phenotype in Tfh cells from both *Il2*^{-/-} and T1D-prone NOD mice. Indeed, the transcriptome of Tfh cells from the PP of NOD mice contained both Tfh and Th17 signature genes that were not expressed in Th cells from the inflamed pancreas. High levels of IL-17 have been previously reported in the colon of young NOD mice, which may reflect GIT inflammation (28). When CCR9⁺ cells were compared with their CCR9⁻ counterparts at each site; CCR9⁺ Tfh cells from the PP and CCR9⁺ Th cells from the pancreas shared genes that were among the most DE at both sites, including *Ccr9*, genes induced by cytokines, interferons and retinoic acid such as *Cxcl9*, *Cx3cr1*, *Aif1*, and *Tnfrsf2* (51), *Lilrb3*, which binds MHC1 to transduce a negative signal (52) and a component of the retinoid receptor *Rarg* (53), which is consistent with the GIT microenvironment where retinoic acid is produced by CD103 expressing dendritic cells (54).

The ligand for CCR9 is Chemokine (C-C motif) ligand 25 (CCL25), which is expressed in the pancreas of pre-diabetic NOD mice (27). Similarly, inflammation can increase the expression of CCL25 in colitis where CCL25 expression correlates with inflammation in the GIT (55). In this manner, the inflamed lesions of the pancreas observed in NOD mice may serve to attract CCR9⁺ Tfh cells from the GIT and GIT-associated lymphoid tissues, or CCR9⁺ Th cells from the GIT that acquire Tfh characteristics in the pancreas or pancreatic lymph nodes, to participate in the destruction of self-tissue in the pancreas and development of autoimmunity.

Data derived from several clinical studies suggest that pharmaceutical CCR9 small-molecule inhibitors have beneficial

effects in patients with CD (56, 57). The observation of organ-specific CCR9-mediated homing of activated T cells to the intestine renders CCR9 a prime therapeutic target to inhibit the recruitment of immune cells whilst avoiding global immune suppression. A greater understanding of the CCR9 expressing Th cells in immunity and autoimmunity will facilitate the generation of new strategies for the treatment of IBD and inflammatory diseases that affect the accessory organs of the digestive system.

MATERIALS AND METHODS

Mice

Female NOD Ltj mice were obtained from ARC, Perth, WA. C57BL/6 *Il2*^{-/-} mice were purchased from Jackson Laboratories (ME, USA) and backcrossed to C57BL/6 to N12.

Immunohistochemistry

Five micrometer sections of paraffin-embedded intestine were conventionally stained with haematoxylin and eosin (H&E) for histological evaluation. Sections were analyzed using a Leica light microscope (Leica Microsystems, Wetzlar, Germany). The images were processed using the Leica acquisition and analysis software ImageJ (Freeware NIH Bethesda, USA) and Adobe Photoshop, version 7 (San José, CA).

Flow Cytometry; Surface Immunostaining

Spleen and lymph nodes were homogenized using 70 μ m cells strainers in lymphocyte isolation buffer. For flow cytometric analysis of pancreas infiltrate, intraepithelial lymphocytes (IELs) and lamina propria lymphocytes (LPLs), small intestine samples were subjected to lymphocyte isolation as described in detail below. Red blood cells (RBC) were removed from spleens using 2 ml RBC lysis buffer for 1 min on ice before washing in lymphocyte isolation buffer. Fifty microliter of a single cell suspension at 2×10^7 cells/ml from spleen and lymph nodes were stained in FACS buffer containing pre-titrated antibodies in 96 well V-bottomed microtitre plates (Nunc, Roskilde, Denmark) at concentrations shown in **Table 1**. To reduce non-specific binding, cells were pre-treated with anti-CD16 for 20 min (2.4G2 made in house). Cells were acquired using Canto cytometer (BD Biosciences, CA) and analyzed using FlowJo (Treestar, CA). Doublets were excluded by forward scatter height and width. Data was collected on a Canto flow cytometer (BD Biosciences), and analyzed using FlowJo software (Tree Star, Inc.).

Flow Cytometry; Intracellular Immunostaining for Detection of Cytokines

Intracellular cytokines were detected using the BD sciences intracellular staining kit according to the manufacturer's instructions. Cytokines were detected either directly *ex-vivo* or after 4 h stimulation at 37°C in cell culture media with PMA (50 ng/ml, BIOMOL), ionomycin (500 ng/ml, Invitrogen) and GolgiPlug (1:1000, BD Biosciences) following *ex vivo* staining of surface markers, cells were fixed and permeated (BD Biosciences), followed by intracellular staining with antibodies at concentrations shown in **Table 1** (58).

FACS Sorting of CCR9⁺ and CCR9⁻ Tfh and Th Cells

For sorting of CCR9⁺/⁻ Tfh and Th cells for microarray analyses (FACSaria cell sorter), we gated on lymphocytes, CD4⁺ T cells by CD4 expression, and then subdivided by CD44 and CCR9. In this way we circumvented staining CD3 by gating lymphocytes to avoid potentially activating the cells being sorted. RNA was extracted from cells with an RNeasy Mini Kit (QIAGEN).

Real-Time qPCR

RNA was obtained from cells with an RNeasy Mini Kit (QIAGEN) and cDNA synthesized with the SuperScript III First-Strand Synthesis System (Life Technologies). We determined the relative abundance of cDNAs in triplicate by qRT-PCR analysis using the Light Cycler 480 (Roche). Fluorescence signals were measured over 45 PCR cycles (94°C for 15 s, 60°C for 30 s, 72°C for 15 s) and the cycle (Ct) at which signals crossed a threshold set within the logarithmic phase was recorded. For each assay, standard curves were generated to identify positive signals on the linear part of the curve. Real-time pre-designed PCR primer pairs for mouse genes were obtained from Applied Biosystems and probes from Roche. Housekeeping gene: Rpl19 F ccacaagctctttctcttcg Roche probe #46, Rpl19 R ggatccaaccagaccttctt Roche probe #46. For quantitative real-time PCR (qRT-PCR), 200 ng RNA was treated with DNase I (Qiagen) for 30 min at 37°C, before cDNA synthesis. Relative

gene expression values were normalized based on the level of the housekeeping gene Rpl19 and calculated using the relative gene quantification tool from the LightCycler 480 software (v.1.5, Roche). Fold modulation of mRNA was calculated by employing a comparative Ct method; Relative abundance of genes = $2^{(\Delta Ct)}$, where ΔCt is the difference between the Ct of target and the arithmetic mean of Cts of Rpl19.

Pancreas Infiltrate Isolation

Mice were perfused with PBS, and pancreas extracted (27). Pancreas were cut into small pieces with scissors and transferred into 50 ml falcon tubes with 3 mls of 0.25 mg/ml Liberase-Enzyme Blend-RI (Roche) in serum free RPMI 1640 media. The tissue was digested in a 37°C water bath for 20 min. Tubes were centrifuged at 201 g at 4°C for 5 min and the supernatant discarded. ten ml of cold serum containing (10%) RPMI 1640 media was added. Tubes were vortexed and shaken to dislodge the tissue; centrifuged at 201 g at 4°C for 5 min and the supernatant discarded. Again the supernatant was discarded, and the tissue resuspended in 5 ml serum-free RPMI 1640, centrifuged at 201 g at 4°C for 5 min. Pellets were thoroughly resuspended in 10 ml histopaque (Sigma-Aldrich) by vortexing. 5 ml of serum-free RPMI 1640 was layered on top. The tubes were centrifuged at 974 g at 4°C for 10 min without rotor acceleration or deceleration Pancreatic infiltrating lymphocytes at the media:histopaque interface were collected, and transferred into a new 15 ml tube. Tubes were the centrifuged at 340 g at 4°C for 5 min, the supernatant discarded, the pellet washed in 5 ml PBS and centrifuged again. To dislodge clumped cells, samples were resuspended in 1 ml x for 1 min and washed in serum containing RPMI 1640.

LPL and IEL Isolation

Small intestines were extracted from mice and placed in a petri dish containing pre-warmed PBS. Peyer's patches were removed and the small intestine was cut longitudinally to allow feces to be washed away. Samples were cut into small pieces with scissors and transferred into 50 ml falcon tubes and washed until media cleared.

To isolate intraepithelial lymphocytes (IEL), the tissues were incubated in 20 ml of IEL stripping buffer for 20 min at 37°C while shaking. Tissues were allowed to settle and the supernatant decanted through a cell strainer, then washed twice in lymphocyte isolation media and suspended in 8 ml of 40% Percoll (GE Healthcare). Three microliters of 70% Percoll was underlayered using a glass pipette and the sample was centrifuged at 600 g for 20 min at room temperature. The IEL were then removed from the resulting interface, washed twice in lymphocyte isolation media by centrifuging at 300 g for 5 min at 4°C and used immediately for flow cytometry analysis.

To isolate the lamina propria lymphocytes (LPL), the tissue remaining after treatment with stripping buffer was washed twice as above and resuspended in 5 ml of 5 mg/ml collagenase D (Roche) and 0.05% DNase (Promega) in lymphocyte isolation media. Tissues were incubated in the enzyme solution for 15 min at 37°C then another 10 mL were added and incubated for another 15 min. Tissues were removed and washed twice as above, then passed through a 70 μ m cell strainer. These cells were

TABLE 1 | Antibodies for Flow Cytometry.

Molecule	Clone (company)	Label	Dilution
CCR6	140706 (BD Biosciences)	Alexa Fluor 647	1:200
CCR9	eBioCW-1.2 (eBioscience)	FITC	1:150
CD122	PO3.1 (eBioscience)	PE	1:100
CD127	A7R34 (eBioscience)	APC	1:200
CD3	145-2C11 (BD Biosciences)	FITC	1:200
		Pacific Blue	1:200
CD4	RM4-5 (BD Biosciences)	APC	1:400
		Alexa Fluor 750	1:300
		Pacific Blue	1:300
CD44	IM7 (eBioscience)	FITC	1:200
		APC	1:200
CD45.2	A20 (eBioscience)	PeCy7	1:300
CD69	H1.2F3 (BD Biosciences)	FITC	1:200
CXCR5	2G8 (BD Biosciences)	Biotin	1:100
IFN γ	XMG1.2 (eBioscience)	FITC	1:200
		Pacific Blue	1:100
IL-10	JES5-16E3 (eBioscience)	APC	1:200
IL-17A	TC11-18H10 (eBioscience)	FITC	1:100
IL-21	Polyclonal (R&D Systems)	Biotin	1:100
IL-22	142928 (R&D Systems)	PE	1:100
IL-4	BVD6-24G2 (eBioscience)	FITC	1:100
IL-9R	224325 (R&D Systems)	PE	1:200
PD-1	J43 (BD Biosciences)	FITC	1:100
		PE	1:100
TNF α	MP6-XT22 (BD Biosciences)	PE	1:200
α 4 β 7	DATK32 (BD Biosciences)	PE	1:200

run on a Percoll gradient as above then used immediately for analysis.

Adoptive Transfer of CCR9⁺/– Tfh and Th Cells

CCR9⁺/– Tfh and Th cells were sorted (FACS Aria cell sorter) for adoptive transfer from combined MLN and Peyer's patches by gating on lymphocytes then CD4⁺ CD44⁺ cells, CCR9⁺ or CCR9[–] cells and CXCR5 to denote Tfh cells. For CFSE labeling, cells were washed twice in PBS and resuspended at 5×10^7 per ml for CFSE staining in CFSE buffer containing 5 mM CFSE. Cells were incubated at 37°C for 10 min then washed twice with ice cold lymphocyte isolation media before being prepared for adoptive transfer. Twelve week-old female NOD mice were administered 6×10^5 (i.v.) of CFSE labeled CCR9⁺ Tfh cells, CCR9[–] Tfh cells, CCR9⁺ Th cells or CCR9[–] Th cells and recovery of CFSE⁺ cells from the MLN, PP and pancreas infiltrate was determined by immunostaining, flow cytometry and FACS analyses on day 4.

Data Analyses and Statistics

P-values were determined by either students *T*-test or 2-way ANOVA using Bonferroni's multiple comparisons test. Data are

reported as the mean $\pm$ standard deviation (SD), along with the calculated *P*-values.

ETHICS STATEMENT

Animals were housed under specific pathogen-free conditions and handled in accordance with the Garvan Institute of Medical Research and St. Vincent's Hospital Animal Experimentation and Ethics Committee, which comply with the Australian code of practice for the care and use of animals for scientific purposes.

AUTHOR CONTRIBUTIONS

IC and HM performed the experiments, analyzed the data, and prepared the figures. JW performed the experiments. MD was involved in discussion about the work and planning of the experiments. CK directed the research, analyzed the data, created the figures, and wrote the manuscript.

FUNDING

This work was supported by Project grant APP1066243 from the National Health and Medical Research Council of Australia.

REFERENCES

- Penhale WJ, Farmer A, McKenna RP, Irvine WJ. Spontaneous thyroiditis in thymectomized and irradiated Wistar rats. *Clin Exp Immunol.* (1973) 15:225–36.
- Penhale WJ, Farmer A, Irvine WJ. Thyroiditis in T cell-depleted rats. Influence of strain, radiation dose, adjuvants and antilymphocyte serum. *Clin Exp Immunol.* (1975) 21:362–75.
- Powrie F, Leach MW, Mauze S, Caddle LB, Coffman RL. Phenotypically distinct subsets of CD4⁺ T cells induce or protect from chronic intestinal inflammation in C. B-17 scid mice. *Int Immunol.* (1993) 5:1461–71. doi: 10.1093/intimm/5.11.1461
- Mottet C, Uhlig HH, Powrie F. Cutting edge: cure of colitis by CD4⁺CD25⁺ regulatory T cells. *J Immunol.* (2003) 170:3939–43. doi: 10.4049/jimmunol.170.8.3939
- Aranda R, Sydora BC, McAllister PL, Binder SW, Yang HY, Targan SR, et al. Analysis of intestinal lymphocytes in mouse colitis mediated by transfer of CD4⁺, CD45RBhigh T cells to SCID recipients. *J Immunol.* (1997) 158:3464–73.
- Podolsky DK. Inflammatory bowel disease. *N Eng J Med.* (2002) 347:417–29. doi: 10.1056/NEJMra020831
- Molodecky NA, Soon IS, Rabi DM, Ghali WA, Ferris M, Chernoff G, et al. Increasing incidence and prevalence of the inflammatory bowel diseases with time, based on systematic review. *Gastroenterology* (2012) 142:46–54 e42; quiz e30. doi: 10.1053/j.gastro.2011.10.001
- Caprilli R, Angelucci E, Cocco A, Viscido A, Zippi M. Efficacy of conventional immunosuppressive drugs in IBD. *Dig Liver Dis.* (2004) 36:766–80. doi: 10.1016/j.dld.2004.06.014
- Duchmann R, Marker-Hermann E, Meyer zum Buschenfelde KH. Bacteria-specific T-cell clones are selective in their reactivity towards different enterobacteria or *H. pylori* and increased in inflammatory bowel disease. *Scand J Immunol.* (1996) 44:71–9. doi: 10.1046/j.1365-3083.1996.d01-273.x
- Wei X, Yang Z, Rey FE, Ridaura VK, Davidson NO, Gordon JI, et al. Fatty acid synthase modulates intestinal barrier function through palmitoylation of mucin 2. *Cell Host Microbe* (2012) 11:140–52. doi: 10.1016/j.chom.2011.12.006
- Goillot E, Mutin M, Touraine JL. Sialadenitis in nonobese diabetic mice: transfer into syngeneic healthy neonates by splenic T lymphocytes. *Clin Immunol Immunopathol.* (1991) 59:462–73. doi: 10.1016/0090-1229(91)90041-8
- Graewin SJ, Kiely JM, Lee K-H, Svatek CL, Nakeeb A, Pitt HA. Nonobese diabetic mice have diminished gallbladder motility and shortened crystal observation time. *J Gastrointest Surg.* (2004) 8:824–9; discussion 829–30. doi: 10.1016/j.gassur.2004.06.014
- Lennon GP, Bettini M, Burton AR, Vincent E, Arnold PY, Santamaria P, et al. T cell islet accumulation in type 1 diabetes is a tightly regulated, cell-autonomous event. *Immunity* (2009) 31:643–53. doi: 10.1016/j.immuni.2009.07.008
- Vaarala O. The gut immune system and type 1 diabetes. *Ann N Y Acad Sci.* (2002) 958:39–46. doi: 10.1111/j.1749-6632.2002.tb02945.x
- Hanninen A, Jaakkola I, Jalkanen S. Mucosal addressin is required for the development of diabetes in nonobese diabetic mice. *J Immunol.* (1998) 160:6018–25.
- Phillips JM, Haskins K, Cooke A. MAdCAM-1 is needed for diabetes development mediated by the T cell clone, BDC-2.5. *Immunology* (2005) 116:525–31. doi: 10.1111/j.1365-2567.2005.02254.x
- Kawamoto S, Tran TH, Maruya M, Suzuki K, Doi Y, Tsutsui Y, et al. The inhibitory receptor PD-1 regulates IgA selection and bacterial composition in the gut. *Science* (2012) 336:485–9. doi: 10.1126/science.1217718
- Kato LM, Kawamoto S, Maruya M, Fagarasan S. Gut Tfh and IgA: key players for regulation of bacterial communities and immune homeostasis. *Immunol Cell Biol.* (2014) 92:49–56. doi: 10.1038/icb.2013.54
- Chen Z, O'Shea JJ. Th17 cells: a new fate for differentiating helper T cells. *Immunol Res.* (2008). doi: 10.1007/s12026-007-8014-9
- Leppkes M, Becker C, Ivanov II, Hirth S, Wirtz S, Neufert C, et al. RORgamma-expressing Th17 cells induce murine chronic intestinal inflammation via redundant effects of IL-17A and IL-17F. *Gastroenterology* (2009) 136:257–67. doi: 10.1053/j.gastro.2008.10.018
- Caruso R, Botti E, Sarra M, Esposito M, Stolfi C, Diluvio L, et al. Involvement of interleukin-21 in the epidermal hyperplasia of psoriasis. *Nat Med.* (2009) 15:1013–5. doi: 10.1038/nm.1995
- King C. New insights into the differentiation and function of T follicular helper cells. *Nat Rev Immunol.* (2009) 9:757–766. doi: 10.1038/nri2644
- Mora JR, Iwata M, Eksteen B, Song SY, Junt T, Senman B, et al. Generation of gut-homing IgA-secreting B cells by intestinal dendritic cells. *Science* (2006) 314:1157–60. doi: 10.1126/science.1132742

24. Papadakis KA, Landers C, Prehn J, Kouroumalis EA, Moreno ST, Gutierrez-Ramos JC, et al. CC chemokine receptor 9 expression defines a subset of peripheral blood lymphocytes with mucosal T cell phenotype and Th1 or T-regulatory 1 cytokine profile. *J Immunol* (2003) 171:159–65. doi: 10.4049/jimmunol.171.1.159
25. Mora JR, Bono MR, Manjunath N, Weninger W, Cavanagh LL, Roseblatt M, et al. Selective imprinting of gut-homing T cells by Peyer's patch dendritic cells. *Nature* (2003) 424:88–93. doi: 10.1038/nature01726
26. Koenecke C, Forster R. CCR9 and inflammatory bowel disease. *Expert Opin Ther Targets* (2009) 13:297–306. doi: 10.1517/14728220902762928
27. McGuire HM, Vogelzang A, Ma CS, Hughes WE, Silveira PA, Tangye SG, Christ D, et al. A subset of interleukin-21+ chemokine receptor CCR9+ T helper cells target accessory organs of the digestive system in autoimmunity. *Immunity* (2011) 34:602–15. doi: 10.1016/j.immuni.2011.01.021
28. Alam C, Valkonen S, Palagani V, Jalava J, Eerola E, Hanninen A. Inflammatory tendencies and overproduction of IL-17 in the colon of young NOD mice are counteracted with diet change. *Diabetes* (2010) 59:2237–46. doi: 10.2337/db10-0147
29. Vogelzang A, McGuire HM, Liu SM, Gloss B, Mercado K, Earls P, et al. IL-21 Contributes to fatal inflammatory disease in the absence of Foxp3(+) T regulatory cells. *J Immunol* (2014) 192:1404–14. doi: 10.4049/jimmunol.1302285
30. Schultz M, Tonkonogy SL, Sellon RK, Veltkamp C, Godfrey VL, Kwon J, Grenther WB, et al. IL-2-deficient mice raised under germfree conditions develop delayed mild focal intestinal inflammation. *Am J Physiol* (1999) 276:G1461–72. doi: 10.1152/ajpgi.1999.276.6.G1461
31. Wollenberg I, Agua-Doce A, Hernandez A, Almeida C, Oliveira VG, Faro J, et al. Regulation of the germinal center reaction by Foxp3+ follicular regulatory T cells. *J Immunol* (2011) 187:4553–60. doi: 10.4049/jimmunol.1101328
32. Chung Y, Tanaka S, Chu F, Nurieva RI, Martinez GJ, Rawal S, et al. Follicular regulatory T cells expressing Foxp3 and Bcl-6 suppress germinal center reactions. *Nat Med* (2011) 17:983–8. doi: 10.1038/nm.2426
33. Vogelzang A, McGuire HM, Yu D, Sprent J, Mackay CR, King C. A fundamental role for interleukin-21 in the generation of T follicular helper cells. *Immunity* (2008) 29:127–37. doi: 10.1016/j.immuni.2008.06.001
34. Nurieva R, Yang XO, Martinez G, Zhang Y, Panopoulos AD, Ma L, et al. Essential autocrine regulation by IL-21 in the generation of inflammatory T cells. *Nature* (2007) 448:480–3. doi: 10.1038/nature05969
35. Strober W, Fuss IJ. Proinflammatory cytokines in the pathogenesis of inflammatory bowel diseases. *Gastroenterology* (2011) 140:1756–67. doi: 10.1053/j.gastro.2011.02.016
36. Hsu HC, Yang P, Wang J, Wu Q, Myers R, Chen J, et al. Interleukin 17-producing T helper cells and interleukin 17 orchestrate autoreactive germinal center development in autoimmune BXD2 mice. *Nat Immunol* (2008) 9:166–75. doi: 10.1038/ni1552
37. Bauquet AT, Jin H, Paterson AM, Mitsdoerffer M, Ho IC, Sharpe AH, et al. The costimulatory molecule ICOS regulates the expression of c-Maf and IL-21 in the development of follicular T helper cells and TH-17 cells. *Nat Immunol* (2009) 10:167–75. doi: 10.1038/ni.1690
38. Reinhardt RL, Liang HE, Locksley RM. Cytokine-secreting follicular T cells shape the antibody repertoire. *Nat Immunol* (2009) 10:385–93. doi: 10.1038/ni1715
39. Nurieva RI, Chung Y, Hwang D, Yang XO, Kang HS, Ma L, et al. Generation of T follicular helper cells is mediated by interleukin-21 but independent of T helper 1, 2, or 17 cell lineages. *Immunity* (2008) 29:138–49. doi: 10.1016/j.immuni.2008.05.009
40. Luthje K, Kallies A, Shimohakamada Y, Belz GT, Light A, Tarlinton DM, et al. The development and fate of follicular helper T cells defined by an IL-21 reporter mouse. *Nat Immunol* (2012) 13:491–8. doi: 10.1038/ni.2261
41. Hanninen A, Taylor C, Streeter PR, Stark LS, Sarte JM, Shizuru JA, et al. Vascular addressins are induced on islet vessels during insulinitis in nonobese diabetic mice and are involved in lymphoid cell binding to islet endothelium. *J Clin Invest* (1993) 92:2509–15. doi: 10.1172/JCI116859
42. Hanninen A, Salmi M, Simell O, Jalkanen S. Mucosa-associated (beta 7-integrinhigh) lymphocytes accumulate early in the pancreas of NOD mice and show aberrant recirculation behavior. *Diabetes* (1996) 45:1173–80. doi: 10.2337/diab.45.9.1173
43. Paronen J, Klemetti P, Kantele JM, Savilahti E, Perheentupa J, Akerblom HK, et al. Glutamate decarboxylase-reactive peripheral blood lymphocytes from patients with IDDM express gut-specific homing receptor alpha4beta7-integrin. *Diabetes* (1997) 46:583–8. doi: 10.2337/diab.46.4.583
44. Savilahti E, Ormala T, Saukkonen T, Sandini-Pohjavuori U, Kantele JM, Arato A, et al. Jejuna of patients with insulin-dependent diabetes mellitus (IDDM) show signs of immune activation. *Clin Exp Immunol* (1999) 116:70–7. doi: 10.1046/j.1365-2249.1999.00860.x
45. Wurbel MA, Philippe JM, Nguyen C, Victorero G, Freeman T, Wooding P, et al. The chemokine TECK is expressed by thymic and intestinal epithelial cells and attracts double- and single-positive thymocytes expressing the TECK receptor CCR9. *Eur J Immunol* (2000) 30:262–71. doi: 10.1002/1521-4141(200001)30:1<262::AID-IMMU262>3.0.CO;2-0
46. Papadakis KA, Prehn J, Moreno ST, Cheng L, Kouroumalis EA, Deem R, Breaverman T, et al. CCR9-positive lymphocytes and thymus-expressed chemokine distinguish small bowel from colonic Crohn's disease. *Gastroenterology* (2001) 121:246–54. doi: 10.1053/gast.2001.27154
47. Apostolaki M, Manoloukos M, Roulis M, Wurbel MA, Muller W, Papadakis KA, et al. Role of beta7 integrin and the chemokine/chemokine receptor pair CCL25/CCR9 in modeled TNF-dependent Crohn's disease. *Gastroenterology* (2008) 134:2025–35. doi: 10.1053/j.gastro.2008.02.085
48. Wang C, Kang SG, Lee J, Sun Z, Kim CH. The roles of CCR6 in migration of Th17 cells and regulation of effector T-cell balance in the gut. *Mucosal Immunol* (2009) 2:173–83. doi: 10.1038/mi.2008.84
49. Rivera-Nieves J, Ho J, Bamias G, Ivashkina N, Ley K, et al. Antibody blockade of CCL25/CCR9 ameliorates early but not late chronic murine ileitis. *Gastroenterology* (2006) 131:1518–29. doi: 10.1053/j.gastro.2006.08.031
50. Saruta M, Yu QT, Avanesyan A, Flesher PR, Targan SR, Papadakis KA. Phenotype and effector function of CC chemokine receptor 9-expressing lymphocytes in small intestinal Crohn's disease. *J Immunol* (2007) 178:3293–300. doi: 10.4049/jimmunol.178.5.3293
51. Wolf FW, Sarma V, Seldin M, Drake S, Suchard SJ, Shao H, et al. Dixit VM, B94, a primary response gene inducible by tumor necrosis factor-alpha, is expressed in developing hematopoietic tissues and the sperm acrosome. *J Biol Chem* (1994) 269:3633–40.
52. Colonna M, Navarro F, Bellon T, Llano M, Garcia P, Samaridis J, et al. A common inhibitory receptor for major histocompatibility complex class I molecules on human lymphoid and myelomonocytic cells. *J Exp Med* (1997) 186:1809–18. doi: 10.1084/jem.186.11.1809
53. Li D, Wang F, Samuels HH. Domain structure of the NRIF3 family of coregulators suggests potential dual roles in transcriptional regulation. *Mol Cell Biol* (2001) 21:8371–84. doi: 10.1128/MCB.21.24.8371-8384.2001
54. Coombes JL, Powrie F. Dendritic cells in intestinal immune regulation. *Nat Rev Immunol* (2008) 8:435–46. doi: 10.1038/nri2335
55. Trivedi PJ, Bruns T, Ward S, Mai M, Schmidt C, Hirschfield GM, et al. Intestinal CCL25 expression is increased in colitis and correlates with inflammatory activity. *J Autoimmun* (2016) 68:98–104. doi: 10.1016/j.jaut.2016.01.001
56. Hart AL, Ng SC, Mann E, Al-Hassi HO, Bernardo D, Knight SC. Homing of immune cells: role in homeostasis and intestinal inflammation. *Inflamm Bowel Dis* (2010) 16:1969–77. doi: 10.1002/ibd.21304
57. Thomas S, Baumgart DC. Targeting leukocyte migration and adhesion in Crohn's disease and ulcerative colitis. *Inflammopharmacology* (2012) 20:1–18. doi: 10.1007/s10787-011-0104-6
58. Jandl C, Loetsch C, King C. Cytokine expression by T follicular helper cells. *Methods Mol Biol* (2017) 1623:95–103. doi: 10.1007/978-1-4939-7095-7_8

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2019 Cosorich, McGuire, Warren, Danta and King. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



PI3K Orchestrates T Follicular Helper Cell Differentiation in a Context Dependent Manner: Implications for Autoimmunity

Silvia Preite^{1,2}, Bonnie Huang^{1,2}, Jennifer L. Cannons^{1,2}, Dorian B. McGavern³ and Pamela L. Schwartzberg^{1,2*}

¹ National Institute of Allergy and Infectious Disease, National Institutes of Health, Bethesda, MD, United States, ² National Human Genome Research Institute, National Institutes of Health, Bethesda, MD, United States, ³ National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, MD, United States

OPEN ACCESS

Edited by:

Shahram Salek-Ardakani,
Pfizer, United States

Reviewed by:

Georges Abboud,
University of Florida, United States

David A. Fruman,
University of California, Irvine,
United States

Laurence Morel,
University of Florida, United States

*Correspondence:

Pamela L. Schwartzberg
pams@nih.gov

Specialty section:

This article was submitted to
T Cell Biology,
a section of the journal
Frontiers in Immunology

Received: 13 September 2018

Accepted: 12 December 2018

Published: 07 January 2019

Citation:

Preite S, Huang B, Cannons JL, McGavern DB and Schwartzberg PL (2019) PI3K Orchestrates T Follicular Helper Cell Differentiation in a Context Dependent Manner: Implications for Autoimmunity. *Front. Immunol.* 9:3079. doi: 10.3389/fimmu.2018.03079

T follicular helper (T_{fh}) cells are a specialized population of CD4⁺ T cells that provide help to B cells for the formation and maintenance germinal centers, and the production of high affinity class-switched antibodies, long-lived plasma cells, and memory B cells. As such, T_{fh} cells are essential for the generation of successful long-term humoral immunity and memory responses to vaccination and infection. Conversely, overproduction of T_{fh} cells has been associated with the generation of autoantibodies and autoimmunity. Data from gene-targeted mice, pharmacological inhibitors, as well as studies of human and mice expressing activating mutants have revealed that PI3K δ is a key regulator of T_{fh} cell differentiation, acting downstream of ICOS to facilitate inactivation of FOXO1, repression of *Klf2* and induction of *Bcl6*. Nonetheless, here we show that after acute LCMV infection, WT and activated-PI3K δ mice (*Pik3cd*^{E1020K/+}) show comparable ratios of T_{fh}:Th1 viral specific CD4⁺ T cells, despite higher polyclonal T_{fh} cells in *Pik3cd*^{E1020K/+} mice. Thus, the idea that PI3K activity primarily drives T_{fh} cell differentiation may be an oversimplification and PI3K-mediated pathways are likely to integrate multiple signals to promote distinct effector T cell lineages. The consequences of dysregulated T_{fh} cell generation will be discussed in the context of the human primary immunodeficiency “Activated PI3K-delta Syndrome” (APDS), also known as “p110 delta-activating mutation causing senescent T cells, lymphadenopathy and immunodeficiency” (PASLI). Overall, these data underscore a major role for PI3K signaling in the orchestration of T lymphocyte responses.

Keywords: T_{fh} cells, T_{fr}, ICOS, PI3K, APDS, PASLI, autoimmunity

INTRODUCTION

Naïve CD4⁺ T helper (Th) cells play pivotal roles in adaptive immunity through the differentiation into distinct cytokine-producing effector subsets that specifically fight a wide range of pathogens and tumors (1). T follicular helper (T_{fh}) cells provide help to B cells for the formation of germinal centers (GCs) (2–4), a specialized microenvironment where clonal expansion of B cells,

immunoglobulin diversification, affinity maturation, and development of memory B and long-lived plasma cells occur in response to immune challenge (5). The outcome of GC reactions requires proper help provided by Tfh cells (6–9). Most successful human vaccines are based on the generation of long-term protective humoral responses derived from the interactions of Tfh and GC B cells; however, Tfh cells can also promote dysregulated responses and autoimmunity (7, 10, 11). It is, therefore, critical to understand factors that promote or limit Tfh cells to elicit tightly controlled GC responses.

Recent data from gene-targeted mice, as well as mice and humans expressing activating mutants of phosphatidylinositol 3-kinase delta (PI3K δ), suggest that PI3K activity is an essential component of pathways driving Tfh cell and GC formation (12–16). In this review, we discuss PI3K δ -mediated pathways involved in the generation, maintenance and function of Tfh cells, including cellular receptors that activate PI3K within T cells, molecular pathways activated, and implications for autoimmunity, with a focus on the genetic disease APDS/PASLI.

PI3K SIGNALING IN IMMUNITY

PI3K Signaling

The PI3Ks are a family of heterodimeric lipid kinases that are activated downstream of a variety of receptors, including growth factor, antigen, costimulatory, cytokine, chemokine, and Toll-like receptors (17, 18). Class IA PI3Ks consist of a p85 regulatory and a p110 catalytic subunit that catalyzes the addition of a phosphate to the membrane phospholipid PI(4,5)P₂, to generate phosphoinositide 3,4,5-triphosphate (PIP₃). PIP₃ helps recruit signaling molecules containing pleckstrin homology and other PIP₃-binding domains to the plasma membrane to propagate signaling cascades (**Figure 1**). Mammals express three class IA catalytic isoforms: the broadly expressed p110 α and p110 β , and p110 δ , which is expressed primarily by immune cells (17). Notably, PI3K δ is activated by a variety of cell-surface receptors that are critical for Tfh cell differentiation, localization and function, including the T-cell receptor, CD28, and ICOS co-receptors, and cytokine receptors (17).

Activated-PI3K coordinates the recruitment of molecules such as PDK1 that phosphorylates and activates the serine/threonine kinase AKT, which in turn phosphorylates multiple targets. Among these are the FOXO transcription factors, which are then sequestered outside the nucleus by 14-3-3 proteins and degraded. FOXOs regulate transcription of multiple genes involved in lymphocyte development, differentiation and function (17, 20). Another downstream effector of PI3K is the mammalian Target of Rapamycin kinase (mTOR), which forms two complexes, mTORC1 and mTORC2, with different scaffolding partners (21). AKT activates mTORC1, an ancient regulator of metabolism, protein synthesis, and cell growth. mTORC2 is essential to fully phosphorylate and activate AKT, thus contributing to downstream signaling, including FOXO1 inactivation, and actin reorganization (21–23). PI3K is counteracted by the lipid-phosphatases PTEN and SHIP-1/2 that convert PIP₃ to PI(4,5)P₂ and PI(3,4)P₂, respectively, (17) (**Figure 1**).

The importance of PI3K δ in lymphocyte function is highlighted by the human primary immunodeficiency APDS/PASLI, in which patients are heterozygous for activating mutations in *PIK3CD*, the gene encoding p110 δ . These patients show immunodeficiency and lymphopenia, as well as lymphoproliferation and autoimmunity (14, 15, 24, 25). Four independent groups, including us, have recently generated mouse models expressing the E1020K activating mutant of p110 δ , which recapitulate many features of APDS/PASLI (16, 26–28). Notably, patients and *Pik3cd*^{E1020K/+} mice exhibit elevated circulating Tfh cells and GCs associated with autoantibody production (15, 16, 24, 29). Mice that express constitutively active p110 α in T cells (30) or have a T cell-specific deletion of PTEN (13) also have elevated Tfh cell frequencies, supporting a more general connection between PI3K activity and Tfh cells. Nonetheless, the observation that p110 δ -inactivation in T cells abrogates Tfh cell generation, supports a non-redundant role of p110 δ in this process (12, 13). Together, these data provide strong evidence that PI3K δ is an important component of pathways driving Tfh cell differentiation.

Tfh Cell Differentiation

The generation of Tfh cells is a multistage process that requires the integration of signals from different cell types (31). In the T cell zone of secondary lymphoid organs, antigen-presenting dendritic cells (DCs) activate T cells to initiate the pre-Tfh cell program, leading to induction of the costimulatory molecule ICOS and chemokine receptor CXCR5, as well as downregulation of CCR7, which together permit migration to the T-B cell border zone (32–34). Here, activated B cells receive signals from pre-Tfh cells to differentiate either along extra-follicular or GC pathways (5, 35). Cognate interactions with activated B cells help promote the differentiation into GC-Tfh cells (36), identified as CXCR5^{hi}PD-1^{hi}Foxp3⁻CD4⁺ T cells that also express high levels of ICOS, CD40L, and the Tfh-master transcription factor BCL-6, which are all critical for Tfh cell differentiation (37, 38). In turn, Tfh cells provide signals via costimulatory molecules and cytokines that help establish and maintain GCs. Thus, the generation of Tfh cells and GC reactions requires intimate communication between T and B cells involving multiple receptors that activate PI3K.

ICOS-PI3K Pathways in Tfh Cells

One of the key costimulatory receptors expressed by Tfh cells is ICOS, a CD28 family member. CD28 and ICOS both activate PI3K δ and are required for Tfh cell development and function. CD28-CD80/CD86 interactions are involved in early T cell activation, including initial induction of ICOS, BCL-6, and CXCR5 (39), which are necessary for Tfh cell formation; *Cd28*^{-/-} mice show a total absence of Tfh cells and thymus-dependent (TD) germinal centers (40–42). ICOS is upregulated on activated T cells shortly after TCR stimulation and interacts with ICOS-ligand (ICOS-L) on antigen presenting cells including DCs and B cells (43). *Icos*^{-/-} and *Icosl*^{-/-} mice display severely reduced humoral response to TD-antigens characterized by a lack of immunological memory and defective GC formation (44–49). Patients lacking ICOS display a common variable immunodeficiency (CVID) with

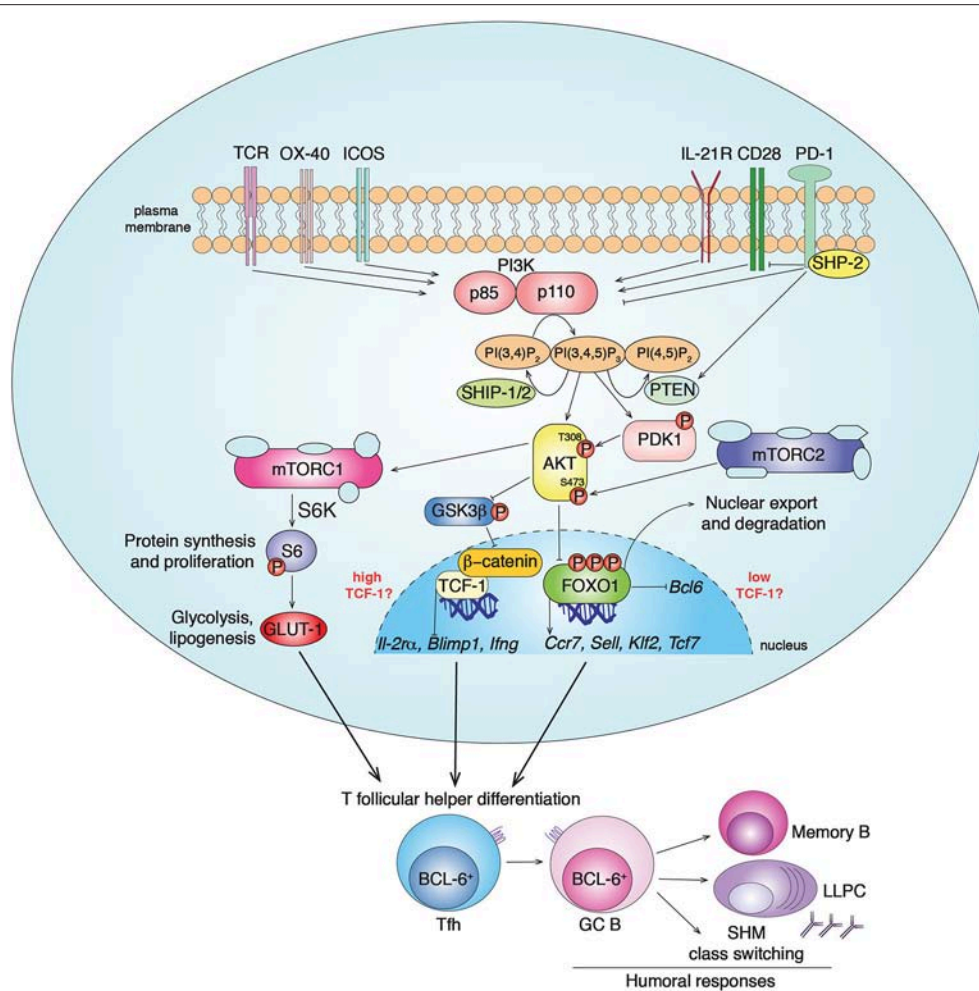


FIGURE 1 | PI3K signaling pathways in Tfh cell differentiation. Class IA PI3Ks (PI3K α , PI3K β , PI3K δ) are lipid kinases composed by a regulatory (p85) and a catalytic (p110) subunit. Multiple receptors activate PI3K in CD4⁺ T cells, including TCR, CD28, ICOS, OX-40 and IL-21R, leading to PI3K recruitment to the plasma membrane and conversion of the membrane lipid PI(4,5)P to PI(3,4,5)P₃. In T cells, chemokine receptors, including CXCR5, preferentially drive the activation of the class IB PI3K γ (19). PI3K activity is counteracted by the inhibitor receptor PD-1 that blocks CD28 signal transduction through SHP-2 recruitment and PTEN induction. The phosphatases PTEN and SHIP-1/2 counteract PI3K signaling by converting PIP₃ to PI(4,5)P₂ and PI(3,4)P₂, respectively. PIP₃ recruits to the plasma membrane proteins containing pleckstrin homology domains, such as AKT and PDK1. The serine/threonine kinase AKT gets activated by phosphorylation by PDK1 (at Thr308) and mTORC2 (at Ser473). In turn, activated pAKT phosphorylates inhibitors of mTORC1 leading to its activation. mTORC1 phosphorylates several factors including S6-kinase (S6K), that phosphorylates S6, driving protein synthesis and cell proliferation, important events for Tfh cell differentiation. pAKT also phosphorylates the FOXO-1 transcription factor leading to its export outside the nucleus and degradation by binding 14-3-3 proteins. FOXO-1 represses *Bcl6*, essential for Tfh cells, and promotes expression of multiple genes including *Sell*, *Tcf7*, *Ccr7*, and *Klf2*. While KLF-2 restrains Tfh cell program through multiple mechanisms, TCF-1 promotes Tfh cell formation by inhibiting *Il-2ra*, *Blimp1*, *Irfng*. At the same time, it has been shown that mTORC2-pAKT may also support TCF-1 activity through the inactivation of GSK3 β , an inhibitor of β -catenin and TCF-1. Overall, PI3K pathways drive BCL-6⁺ Tfh cell differentiation that coordinates GC responses and humoral immunity after infections and vaccination through the generation of memory B cells and long-lived plasma cells (LLPC).

reduced circulating Tfh cells (50, 51). Conversely, mutations affecting Roquin, a negative post-transcriptional regulator of ICOS mRNA, increase Tfh cells and drive autoimmunity (52, 53).

ICOS helps drive multiple stages of Tfh cell differentiation, including the early generation of CXCR5^{high} T cells, modulation of other chemokine and homing receptors through regulation of the KLF2 transcription factor (39, 54), and T:B cell non-cognate interactions that promote T cell motility at the T:B cell border (55). ICOS-ICOS-L interactions are also critical for localization and maintenance of GC-Tfh cells (9, 39, 54).

The essential role of PI3K in ICOS function was highlighted by data showing that mutation of the p85-binding site, which selectively abrogates PI3K recruitment, led to defects in Tfh cell formation similar to ICOS-deficiency (56). Inhibition of p110 δ also prevented ICOS-mediated changes in cell migration and morphology *in vitro* (55). Conversely, we found that activated-PI3K δ mice show T cell-intrinsic increases in Tfh cell differentiation, even in the presence of blocking anti-ICOS-L antibody, therefore bypassing the requirement for ICOS for Tfh cell development (16). Thus, PI3K appears to be a major effector of ICOS, required for Tfh cell formation and maintenance.

PI3K Signaling Downstream of ICOS

After ICOS ligation, activated-PI3K δ transduces its signals through several intermediates, including pAKT-mediated inactivation of FOXO1 (20). FOXO1 transcriptionally represses *Bcl6*, a driver of Tfh cell differentiation (30, 57, 58); strong PI3K activity relieves this repression. FOXO1 also transcriptionally activates *Klf2* (59), which restrains Tfh cells and promotes alternative T helper subsets through at least four mechanisms: (1) induction of *S1pr1*, downregulation of which is essential for Tfh cell retention in GC and efficient polarization; (2) induction of BLIMP1, which negatively regulates *Bcl6* and Tfh cell generation; (3) induction of T-bet and GATA3 which drives Th1 and Th2 cell differentiation, respectively; and (4) repression of *Cxcr5* (39, 60). Accordingly, *Foxo1*^{-/-} CD4⁺ T cells generate increased percentages of pre-Tfh cells (CXCR5^{int}BCL-6^{int}) early post-immunization, even in the presence of anti-ICOS-L (57), similar to cells expressing activated-PI3K δ (16). These data support the ICOS-PI3K δ -FOXO1 pathway as critical for Tfh cell development; accordingly, *Pik3cd*^{E1020K/+} CD4⁺ T cells exhibit elevated pFOXO1 upon TCR stimulation, even without further ICOS re-stimulation (16). Furthermore, an AKT-resistant mutant of FOXO1 prevents increased Tfh cells in the presence of activated-PI3K δ (16). It is also of note that ICOS is a stronger inducer of PI3K than CD28, resulting in greater inhibition of FOXO1; this may account for the inability of CD28 to compensate for ICOS-deficiency in promoting Tfh cells (39, 42, 56, 61, 62). However, *Foxo1*^{-/-} T cells show defective GC-Tfh (CXCR5^{high}BCL-6^{high}) cell formation (57) which is not observed with activated-PI3K δ (16). Thus, cells expressing activated-PI3K δ likely still retain some FOXO1 activity. FOXO1 is required for sustained surface ICOS expression (57), providing a possible explanation for this defect. Indeed, chromatin immunoprecipitation and deep sequencing revealed FOXO1 binding sites in multiple genes that influence Tfh cell fate, including *Cxcr4*, *Batf*, *Irf4*, *Icos*, and *Prdm1* (57, 63).

Nonetheless, despite increased GC-Tfh cell differentiation, *Pik3cd*^{E1020K/+} mice show disorganized GCs with increased Tfh cell infiltration and impaired class-switched antigen-specific responses to immunization (16, 27, 28). Multiple factors may contribute to these poor responses, including impaired B cell selection due to increased Tfh cells (7) and Tfh cell mislocalization (16), or intrinsic B cell defects. Indeed, although deletion of p110 δ in B cells only minimally affected GC formation and T cell-dependent humoral responses after protein immunization (13), activated-PI3K δ drove B cell-intrinsic increases in GC B and plasma cells, as well as impaired class-switched antibody production (16, 28). Increased GC B cells may in turn further drive expanded Tfh cell numbers, contributing to immune dysregulation. Additionally, increased GCs that fill the follicular dendritic cell network at baseline, may prevent new GC formation as mice age (16). Whether and how FOXO1 contributes to defects in antigen-specific responses or whether additional downstream effectors of PI3K are involved remain intriguing questions. It should also be noted that additional receptors expressed by Tfh cells, including OX-40, and IL-21R activate PI3K (64) and may contribute to expanded Tfh cell populations in these mice; in contrast, PD-1, an inhibitory

receptor highly expressed by Tfh cells (65), counteracts PI3K by blocking CD28 signaling and increasing PTEN expression (66–69) (Figure 1).

CONTEXT-DEPENDENT ROLES FOR PI3K IN T CELL DIFFERENTIATION

Viral Infection

Although the connection between ICOS and PI3K δ provides strong evidence for PI3K δ driving Tfh cells, the view that PI3K exclusively promotes Tfh cells may be a simplification; this is particularly apparent when looking at the differentiation of Tfh vs. Th1 cells during viral infection. In response to viral or strong Th1 polarizing infections, CD4⁺ T cells undergo an early bifurcation such that up to 50% of viral-specific T cells express BCL-6 and become Tfh cells, while the rest express BLIMP1 and SLAMF, and become IFN γ -producing Th1 cells (54). Although activated-PI3K δ increased percentages of Tfh cells at baseline and in response to immunization (16), as well as polyclonal CXCR5⁺PD-1⁺ Tfh cells after LCMV infection (Figure 2A), it did not alter Tfh cell percentages, nor Tfh/Th1 ratios, within viral-specific GP66-tetramer⁺ CD4⁺ T cells in the same mice (Figures 2B,C). We also observed increased percentages of circulating CXCR3⁺ Tfh1 cells in patients with APS/PASLI compared to controls (16), suggesting that PI3K can drive both Tfh cells and Type 1 immunity. Thus, PI3K activity may promote multiple effector T cell lineages and the effects of PI3K on Tfh cells may depend on the activating stimuli and microenvironment.

IL2 Signaling

Among potential PI3K-mediated signaling pathways that influence Tfh and Th1 cell differentiation are those downstream from the cytokine IL-2. Early data suggested that PI3K is activated by the IL-2R signaling complex (71–73); PI3K inhibitors arrest IL-2 induced CTL growth (74, 75). However, recent reports question the direct connection between IL-2 and PI3K activation (76), as that: (1) certain PI3K inhibitors (such as LY294002) have off-target effects (77); (2) many studies evaluate pAKT^{S473} and pS6, rather than pAKT^{T308}, which more accurately reflects PI3K activity (78); and (3) IL-2 can promote mTORC1 activation independent of PI3K (79). Indeed, IL-2 potentially inhibits Tfh cell generation via STAT5-mediated induction of BLIMP1 (80–82); BLIMP1⁺ Th1 cells express high levels of the high-affinity IL-2 receptor, CD25, and pSTAT5. As that IL-2 activates multiple signaling pathways, the integration, kinetics, and balance of these and other signals elicited in response to multiple receptors, may ultimately help determine T helper cell fates.

Metabolic Pathways in Tfh vs. Th1 Cells

Other PI3K-mediated signaling pathways that may influence both Tfh and Th1 cells are those involving mTORC1 and mTORC2. During acute LCMV infection, Th1 cells appear more proliferative and bio-energetically demanding with greater glucose metabolism and metabolic respiration than Tfh cells (83). Data suggest that these Th1 cells were more dependent

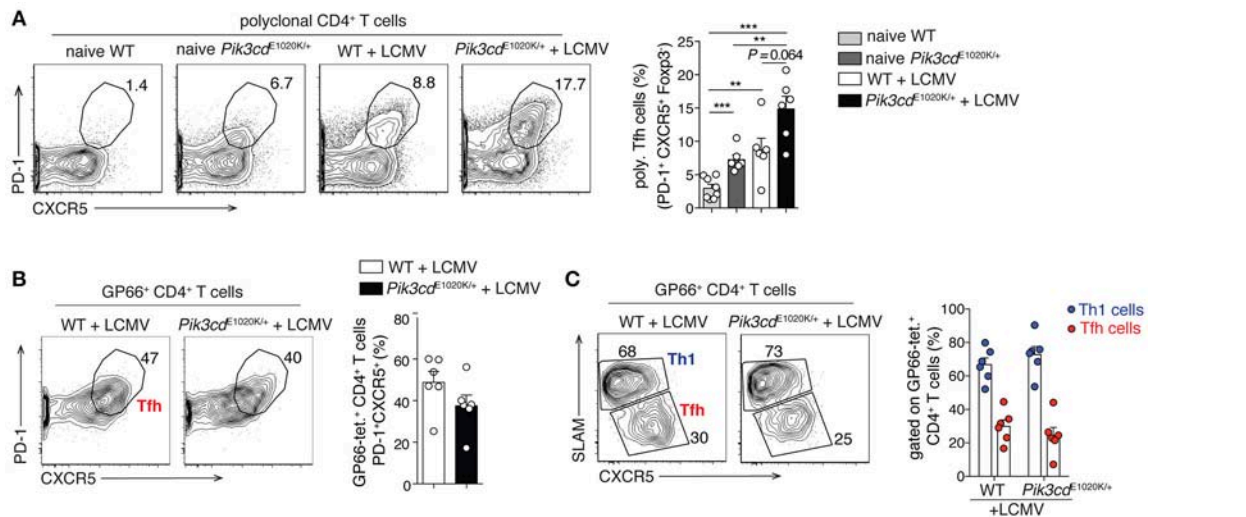


FIGURE 2 | LCMV infection promotes comparable Th1/Tfh cell differentiation within GP66-tetramer⁺ T cells, despite increased polyclonal Tfh cells in *Pik3cd*^{E1020K/+} mice. 2/3-month-old WT and *Pik3cd*^{E1020K/+} mice were infected i.v. with LCMV Arm and analyzed in the spleen 7/8 days post infection. Naive WT and *Pik3cd*^{E1020K/+} mice were analyzed as control. **(A)** Representative contour plots and summary histogram of polyclonal (GP66 tetramer negative) PD-1⁺CXCR5⁺Foxp3⁺ Tfh cells (percentage of CD4⁺B220[−]GP66[−] T cells). **(B)** Representative contour plot and summary histogram of Tfh cells (PD-1⁺CXCR5⁺), gated on GP66-tetramer⁺ CD4⁺B220[−]Foxp3[−] T cells. **(C)** Representative contour plot and summary histogram of Th1 (SLAMF^{hi}CXCR5^{lo}) and Tfh (SLAMF^{lo}CXCR5^{hi}) cells (gated on GP66-tetramer⁺CD4⁺B220[−]Foxp3[−] T cells). We were unable to evaluate GP66⁺ Tfr cells due to the low numbers of GP66⁺ Foxp3⁺ cells (70). However, polyclonal Tfr cells were increased after LCMV infection (data not shown), as we have previously reported in naive mice (16). **(A–C)**, *n* = 5–8. Data are representative of three independent experiments and are expressed as mean ± SEM with each dot indicating one mouse. Significance analyzed by Mann-Whitney *U*-test. ** *p* < 0.01; *** *p* < 0.001.

on the IL-2-PI3K-AKT-mTORC1 axis, which preferentially promoted BLIMP1⁺ Th1 cells at the expense of BCL-6⁺ Tfh cells and humoral immunity (83, 84). However, other studies have demonstrated requirements for mTORC1 and mTORC2 in driving Tfh cells in Peyer's Patches at steady state and in the periphery after LCMV infection and immunization (30, 85). Mechanistically, Tfh cells were supported by mTORC1-promotion of pS6, GLUT1 expression, glycolysis, lipogenesis and overall proliferation; and by mTORC2-pAKT, which decreased FOXO1 activity (30).

While these studies provide conflicting conclusions on the requirements for PI3K and downstream effectors for Tfh cells, this may result from different experimental systems (knockdown vs. knockout) as well as bio-energetic demands during immune challenges. However, there is also evidence that mTOR may be activated independently of PI3K via pathways involving nutrient sensing that may also affect T helper cell differentiation (22, 79, 86, 87).

PI3K-TCF-1 Cross-Talk

Several recent studies revealed that the transcription factor TCF-1 is expressed at high levels in Tfh cells after viral infection and plays an essential role in their generation and maintenance, via repression of *Il2ra*, and *Prdm1* (which encodes BLIMP1), promotion of *Bcl6* (55, 88–90), and possibly repression of *Ifng* (91). Intriguingly, PI3K has been implicated both positively and negatively in TCF-1 regulation (92, 93). In CD8⁺ T cells, *Tcf7*, which encodes for TCF-1, is induced by FOXO1 (94), and both

are required for memory T cell formation (95–98). Strong PI3K signaling would therefore be expected to decrease TCF-1 levels (25), as observed in studies of asymmetric cell division (92, 93). Conversely, a positive link between PI3K/AKT and TCF-1 has been proposed via β -catenin (85), a coactivator of TCF-1 that is negatively regulated by phosphorylation by Glycogen Synthase Kinase 3 β (GSK3 β), which is inactivated by pAKT (**Figure 1**). mTORC2-deficient T cells, which do not fully activate AKT, show reduced β -catenin and TCF-1 (85). Nonetheless, most studies implicating TCF-1 in Tfh cell generation have been done in the context of strong Th1-inducing infections (88–90), and how these findings relate to Tfh cells in other contexts remains unknown. Thus, the relationship between PI3K and TCF-1, how they affect Tfh cell differentiation, and involvement in possible feedback loops remain intriguing questions.

PI3K PATHWAYS IN Tfr CELLS

A subset of thymic derived T regulatory cells, defined as T follicular regulatory (Tfr) cells, are localized at the T-B cell border and inside the GC area (99), and directly control the activation and differentiation of Tfh and GC B cells, including the development of autoimmunity (100). Tfr cells are phenotypically similar to Tfh cells and express BCL-6 (101–103), yet lack expression of B-cell-helper molecules, such as CD40L, and express inhibitory molecules CTLA-4, GITR, IL-10, and granzymes (104). Although excessive PI3K-mTOR activity is detrimental for induced-Treg cell differentiation (105–107),

Pik3cd^{E1020K/+} mice show increased Treg and Tfr cells at steady state (16, 26). Indeed, although Tfr cells derive from Tregs, Tfr have different requirements for their differentiation and function. For example, IL-2 is necessary for Treg development and suppressive capability (108), yet prevents Tfr cell differentiation in a BLIMP1-dependent manner, similar to IL-2's effects on Tfh cells (109). Tfr cells also display high mTORC1 activity that promotes differentiation and STAT3 phosphorylation, which induces *Tcf7* and *Bcl6* (110); increased AKT-mTOR activity in Roquin-deficient Treg cells upregulates Tfh cell gene signatures that drive Tfr cell differentiation (111). How PI3K affects ratios of Tfh:Tfr cells, which are important for regulating humoral responses and autoimmunity (104), is less clear; notably, *Pik3cd*^{E1020K/+} mice have parallel increases in both cell populations (16).

DYSREGULATED PI3K PATHWAYS IN AUTOIMMUNITY

In addition to helping antigen-specific humoral responses to vaccination and infection, Tfh cells have been linked to autoimmunity in both animals and humans (10, 112). Correlations between circulating Tfh (cTfh) cells and disease have been reported in systemic lupus erythematosus (SLE) (113), rheumatoid arthritis (RA) and Sjögren's syndrome (112). Similarly, APDS/PASLI patients have high cTfh cells (16), and autoimmune manifestations including autoantibodies, cytopenias and glomerulonephritis (15, 29). In parallel, we found that *Pik3cd*^{E1020K/+} mice develop autoantibodies against a wide range of self-antigens. Indeed, PIDs caused by mutations affecting PI3K signaling cascades, or "immune TOR-opathies," often display a combination of defective immune-responses and autoimmunity (114); animal models demonstrate that PI3K activity in B cells, T helper and regulatory T cells contributes to autoimmune manifestations (16, 115–121). Additionally, increased PI3K activity has been observed in several autoimmune diseases (121), and inhibitors of PI3K δ and PI3K γ are currently being explored in pre-clinical models of RA and SLE, and clinically for psoriasis (NCT02303509) and Sjögren's (NCT02775916) (121, 122).

Recent data suggest that certain autoantibodies cross-react with gut microbiota, supporting links between the microbiome and autoimmunity (123). Interestingly, we found increased local and systemic immune responses against gut commensals in *Pik3cd*^{E1020K/+} mice, with evidence for cross-reactivity between anti-self and anti-bacterial antibodies. Furthermore, autoantibodies could be prevented by systemic antibiotic treatment (16). Such data highlight roles for PI3K δ in modulating T and B lymphocyte activation, including that induced by the microbiota, which can lead to autoimmunity.

CONCLUDING REMARKS

Together, a growing body of evidence supports a connection between PI3K δ and Tfh cell differentiation, raising the possibility that altered PI3K pathways may contribute to

both immunodeficiency and autoimmunity. Nonetheless, results during viral infection suggest that effects of PI3K on Tfh cell differentiation may be context-dependent and that PI3K may promote multiple effector cell lineages. A recent report demonstrated that treatment of APDS/PASLI patients with leniolisib, a selective PI3K δ inhibitor, showed promising improvements in cellular dysfunction and lympho-proliferation (124). Notably, selective PI3K δ inhibition reduced serum IgM *in vivo* (124), while increasing IgG class-switching *in vitro* (16, 27); however, effects on Tfh cells and autoantibodies have not yet been reported. Our results further suggest that evaluation of microbiota composition and systemic responses to gut commensals in APDS/PASLI may provide new opportunities, possibly in association with leniolisib, for managing this and other conditions where Tfh cells and autoantibodies contribute to pathogenesis. Such approaches may also be relevant for autoimmunity induced by checkpoint-blockade therapy, where PI3K δ -inhibition may provide a selective control of immune responses. Finally, PI3K δ activation may help improve vaccine responses, although this would have to be carefully assessed. Thus, a more comprehensive understanding of PI3K regulation and signaling in T and B cells is of crucial importance to more effectively improve humoral immune responses while minimizing autoimmunity.

METHODS

Animal Care and Ethics

Control (C57Bl/6J) and *Pik3cd*^{E1020K/+} mice (16) were maintained and treated under specific pathogen-free (SPF) conditions under protocols reviewed and approved by the NINDS (protocol 1295-12) and NHGRI (protocol G98-3) Animal Care and Use Committees at the NIH.

LCMV Infection and Flow-Cytometry

Mice were injected intravenously (i.v.) with 2×10^5 plaque-forming units (PFUs) of LCMV Armstrong, kindly provided by Dorian McGavern Lab, grown as previously described (88). Day+7/8 post infection, single cell suspensions were prepared from spleen in MACS buffer (PBS with 2% FBS and 2 μ M EDTA). GP66 tetramer [I-A(b) QVYSLIRPNENPAHK PE] was obtained from NIH tetramer facility (Emory University); staining was performed at 37°C for 1 h in RPMI with 10% serum. CXCR5 staining was performed using: CXCR5-purified (2G8, BD Biosciences), followed by Biotin-SP AffiniPure Fab Fragment Goat Anti-Rat IgG (H+L) (Jackson ImmunoResearch), and Streptavidin (BioLegend) as previously described (88). The following antibodies (obtained from BioLegend, BD Biosciences, eBioscience) were incubated with spleen cells for 45/60 min on ice: CD4 (RM4-5), B220 (RA3-6B2), PD-1 (RMP1-30), SLAM (TC15-12F12.2). Intracellular staining of Foxp3 (FJK-16s) was performed using the Foxp3-staining buffer (eBioscience). Cells were gated according to FSC-A/SSC-A, doublet exclusion (SSC-H/SSC-W and FSC-H/FSC-W), live cells (negative for LIVE/DEAD® Fixable Aqua Dead Cell Stain Kit, Life Technologies), followed by gating strategies indicated in figure legend. Flow cytometry was performed on a LSRII (BD

Biosciences) and data analyzed using FlowJo 9.9 software (TreeStar).

Statistical Analysis

Data were analyzed via Prism 6 (GraphPad Software) using non-parametric unpaired Mann-Whitney *U*-test. Graphs show the mean $\pm$ SEM. **P* < 0.05; ** *P* < 0.01; *** *P* < 0.001. If not indicated, the *P*-values were not significant (>0.05).

AUTHOR CONTRIBUTIONS

SP designed and performed experiments, analyzed and interpreted data, wrote the manuscript and prepared the figures. BH and JLC performed experiments, edited the manuscript, and contributed to discussions. DBM contributed essential reagents and advice. PLS conceived the project, wrote the manuscript,

and provided overall direction of the study. All authors concur with this submission.

FUNDING

This work was supported in part by funds from the intramural programs of the National Human Genome Research Institute, National Institute of Allergy and Infectious Diseases and National Institute of Neurological Disorders and Stroke, NIH.

ACKNOWLEDGMENTS

We thank members of the PLS and DBM labs for their assistance with this work. GP66 tetramer [I-A(b) QVYSLIRPNENPAHK PE] was obtained from the NIH tetramer facility (Emory University).

REFERENCES

- Sallusto F. Heterogeneity of human CD4(+) T cells against microbes. *Annu Rev Immunol.* (2016) 34:317–34. doi: 10.1146/annurev-immunol-032414-112056
- Vinuesa CG, Linterman MA, Goodnow CC, Randall KL. T cells and follicular dendritic cells in germinal center B-cell formation and selection. *Immunol Rev.* (2010) 237:72–89. doi: 10.1111/j.1600-065X.2010.00937.x
- Crotty S. Follicular helper CD4 T cells (TFH). *Annu Rev Immunol.* (2011) 29:621–63. doi: 10.1146/annurev-immunol-031210-101400
- Cannons JL, Lu KT, Schwartzberg PL. T follicular helper cell diversity and plasticity. *Trends Immunol.* (2013) 34:200–7. doi: 10.1016/j.it.2013.01.001
- Mesin L, Ersching J, Vitoria GD. Germinal center B cell dynamics. *Immunity* (2016) 45:471–482. doi: 10.1016/j.immuni.2016.09.001
- Pratama A, Vinuesa CG. Control of TFH cell numbers: why and how? *Immunol Cell Biol.* (2014) 92:40–8. doi: 10.1038/icb.2013.69
- Preite S, Baumjohann D, Foglierini M, Basso C, Ronchi F, Fernandez Rodriguez BM, et al. Somatic mutations and affinity maturation are impaired by excessive numbers of T follicular helper cells and restored by Treg cells or memory T cells. *Eur J Immunol.* (2015) 45:3010–21. doi: 10.1002/eji.201545920
- Brink R, Phan TG. Self-reactive B cells in the germinal center reaction. *Annu Rev Immunol.* (2018) 36:339–57. doi: 10.1146/annurev-immunol-051116-052510
- Baumjohann D, Preite S, Reboldi A, Ronchi F, Ansel KM, Lanzavecchia A, et al. Persistent antigen and germinal center B cells sustain T follicular helper cell responses and phenotype. *Immunity* (2013) 38:596–605. doi: 10.1016/j.immuni.2012.11.020
- Ueno H. T follicular helper cells in human autoimmunity. *Curr Opin Immunol.* (2016) 43:24–31. doi: 10.1016/j.coi.2016.08.003
- Brink R. The imperfect control of self-reactive germinal center B cells. *Curr Opin Immunol.* (2014) 28:97–101. doi: 10.1016/j.coi.2014.03.001
- Okkenhaug K, Bilancio A, Farjot G, Priddle H, Sancho S, Peskett E, et al. Impaired B, and T cell antigen receptor signaling in p110delta PI 3-kinase mutant mice. *Science* (2002) 297:1031–4. doi: 10.1126/science.1073560
- Rolf J, Bell SE, Kovacs D, Janas ML, Soond DR, Webb LM, et al. Phosphoinositide 3-kinase activity in T cells regulates the magnitude of the germinal center reaction. *J Immunol.* (2010) 185:4042–52. doi: 10.4049/jimmunol.1001730
- Angulo I, Vadas O, Garçon F, Banham-Hall E, Plagnol V, Leahy TR, et al. Phosphoinositide 3-kinase delta gene mutation predisposes to respiratory infection and airway damage. *Science* (2013) 342:866–71. doi: 10.1126/science.1243292
- Lucas CL, Kuehn HS, Zhao F, Niemela JE, Deenick EK, Palendira U, et al. Dominant-activating germline mutations in the gene encoding the PI(3)K catalytic subunit p110delta result in T cell senescence and human immunodeficiency. *Nat Immunol.* (2014) 15:88–97. doi: 10.1038/ni.2771
- Preite S, Cannons JL, Radtke AJ, Vujkovic-Cvijin I, Gomez-Rodriguez J, Volpi S, et al. Hyperactivated PI3Kdelta promotes self and commensal reactivity at the expense of optimal humoral immunity. *Nat Immunol.* (2018) 19:986–1000. doi: 10.1038/s41590-018-0182-3
- Okkenhaug K, Vanhaesebroeck B. PI3K in lymphocyte development, differentiation and activation. *Nat Rev Immunol.* (2003) 3:317–30. doi: 10.1038/nri1056
- Fruman DA, Chiu H, Hopkins BD, Bagrodia S, Cantley LC, Abraham RT. The PI3K Pathway in Human Disease. *Cell* (2017) 170:605–35. doi: 10.1016/j.cell.2017.07.029
- Reif K, Okkenhaug K, Sasaki T, Penninger JM, Vanhaesebroeck B, Cyster JG. Cutting edge: differential roles for phosphoinositide 3-kinases, p110gamma and p110delta, in lymphocyte chemotaxis and homing. *J Immunol.* (2004) 173:2236–40. doi: 10.4049/jimmunol.173.4.2236
- Hedrick SM, Hess Michelini R, Doedens AL, Goldrath AW, Stone EL. FOXO transcription factors throughout T cell biology. *Nat Rev Immunol.* (2012) 12:649–61. doi: 10.1038/nri3278
- Saxton RA, Sabatini DM. mTOR Signaling in growth, metabolism, and disease. *Cell* (2017) 168:960–76. doi: 10.1016/j.cell.2017.02.004
- Powell JD, Pollizzi KN, Heikamp EB, Horton MR. Regulation of immune responses by mTOR. *Annu Rev Immunol.* (2012) 30:39–68. doi: 10.1146/annurev-immunol-020711-075024
- Blenis J. TOR, the gateway to cellular metabolism, cell growth, and disease. *Cell* (2017) 171:10–13. doi: 10.1016/j.cell.2017.08.019
- Crank MC, Grossman JK, Moir S, Pittaluga S, Buckner CM, Kardava L, et al. Mutations in PIK3CD can cause hyper IgM syndrome (HIGM) associated with increased cancer susceptibility. *J Clin Immunol.* (2014) 34:272–6. doi: 10.1007/s10875-014-0012-9
- Cannons JL, Preite S, Kapnick SM, Uzel G, Schwartzberg PL. Genetic defects in phosphoinositide 3-kinase delta influence CD8(+) T cell survival, differentiation, and function. *Front Immunol.* (2018) 9:1758. doi: 10.3389/fimmu.2018.01758
- Stark AK, Chandra A, Chakraborty K, Alam R, Carbonaro V, Clark J, et al. PI3Kdelta hyper-activation promotes development of B cells that exacerbate *Streptococcus pneumoniae* infection in an antibody-independent manner. *Nat Commun.* (2018) 9:3174. doi: 10.1038/s41467-018-05674-8
- Avery DT, Kane A, Nguyen T, Lau A, Nguyen A, Lenthall H, et al. Germline-activating mutations in PIK3CD compromise B cell development and function. *J Exp Med.* (2018) 215:2073–95. doi: 10.1084/jem.20180010
- Wray-Dutra MN, Al Qureshi F, Metzler G, Oukka M, James RG, Rawlings DJ. Activated PIK3CD drives innate B cell expansion yet limits B cell-intrinsic immune responses. *J Exp Med.* (2018) 215:2485–96. doi: 10.1084/jem.20180617

29. Coulter TI, Chandra A, Bacon CM, Babar J, Curtis J, Screaton N, et al. Clinical spectrum and features of activated phosphoinositide 3-kinase delta syndrome: a large patient cohort study. *J Allergy Clin Immunol.* (2017) 139:597–606 e4. doi: 10.1016/j.jaci.2016.06.021
30. Zeng H, Cohen S, Guy C, Shrestha S, Neale G, Brown SA, et al. mTORC1 and mTORC2 kinase signaling and glucose metabolism drive follicular helper T cell differentiation. *Immunity* (2016) 45:540–54. doi: 10.1016/j.immuni.2016.08.017
31. Qi H. T follicular helper cells in space-time. *Nat Rev Immunol.* (2016) 16:612–25. doi: 10.1038/nri.2016.94
32. Goenka R, Barnett LG, Silver JS, O'Neill PJ, Hunter CA, Cancro MP, et al. Cutting edge: dendritic cell-restricted antigen presentation initiates the follicular helper T cell program but cannot complete ultimate effector differentiation. *J Immunol.* (2011) 187:1091–5. doi: 10.4049/jimmunol.1100853
33. Baumjohann D, Okada T, Ansel KM. Cutting edge: distinct waves of BCL6 expression during T follicular helper cell development. *J Immunol.* (2011) 187:2089–92. doi: 10.4049/jimmunol.1101393
34. Allen CD, Ansel KM, Low C, Lesley R, Tamamura H, Fujii N, et al. Germinal center dark and light zone organization is mediated by CXCR4 and CXCR5. *Nat Immunol.* (2004) 5:943–52. doi: 10.1038/ni1100
35. MacLennan IC, Toellner KM, Cunningham AF, Serre K, Sze DM, Zuniga E, et al. Extrafollicular antibody responses. *Immunol Rev.* (2003) 194:8–18. doi: 10.1034/j.1600-065X.2003.00058.x
36. Ma CS, Deenick EK, Batten M, Tangye SG. The origins, function, and regulation of T follicular helper cells. *J Exp Med.* (2012) 209:1241–53. doi: 10.1084/jem.20120994
37. Crotty S. T follicular helper cell differentiation, function, and roles in disease. *Immunity* (2014) 41:529–42. doi: 10.1016/j.immuni.2014.10.004
38. Cannons JL, Tangye SG, Schwartzberg PL. SLAM family receptors and SAP adaptors in immunity. *Annu Rev Immunol.* (2011) 29:665–705. doi: 10.1146/annurev-immunol-030409-101302
39. Weber JP, Fuhrmann F, Feist RK, Lahmann A, Al Baz MS, Gentz LJ, et al. ICOS maintains the T follicular helper cell phenotype by down-regulating Kruppel-like factor 2. *J Exp Med.* (2015) 212:217–33. doi: 10.1084/jem.20141432
40. Mittrucker HW, Kohler A, Mak TW, Kaufmann SH. Critical role of CD28 in protective immunity against *Salmonella typhimurium*. *J Immunol.* (1999) 163:6769–76.
41. Shahinian A, Pfeffer K, Lee KP, Kundig TM, Kishihara K, Wakeham A, et al. Differential T cell costimulatory requirements in CD28-deficient mice. *Science* (1993) 261:609–12. doi: 10.1126/science.7688139
42. Linterman MA, Rigby RJ, Wong R, Silva D, Withers D, Anderson G, et al. Roquin differentiates the specialized functions of duplicated T cell costimulatory receptor genes CD28 and ICOS. *Immunity* (2009) 30:228–41. doi: 10.1016/j.immuni.2008.12.015
43. Hutloff A, Dittrich AM, Beier KC, Eljaschewitsch B, Kraft R, Anagnostopoulos I, et al. ICOS is an inducible T-cell co-stimulator structurally and functionally related to CD28. *Nature* (1999) 397:263–6. doi: 10.1038/16717
44. Tafuri A, Shahinian A, Bladt F, Yoshinaga SK, Jordana M, Wakeham A, et al. ICOS is essential for effective T-helper-cell responses. *Nature* (2001) 409:105–9. doi: 10.1038/35051113
45. Mak TW, Shahinian A, Yoshinaga SK, Wakeham A, Boucher LM, Pinter M, et al. Costimulation through the inducible costimulator ligand is essential for both T helper and B cell functions in T cell-dependent B cell responses. *Nat Immunol.* (2003) 4:765–72. doi: 10.1038/ni947
46. Gonzalo JA, Tian J, Delaney T, Corcoran J, Rottman JB, Lora J, et al. ICOS is critical for T helper cell-mediated lung mucosal inflammatory responses. *Nat Immunol.* (2001) 2:597–604. doi: 10.1038/89739
47. Dong C, Temann UA, Flavell RA. Cutting edge: critical role of inducible costimulator in germinal center reactions. *J Immunol.* (2001) 166:3659–62. doi: 10.4049/jimmunol.166.6.3659
48. McAdam AJ, Greenwald RJ, Levin MA, Chernova T, Malenkovich N, Ling V, et al. ICOS is critical for CD40-mediated antibody class switching. *Nature* (2001) 409:102–5. doi: 10.1038/35051107
49. Akiba H, Takeda K, Kojima Y, Usui Y, Harada N, Yamazaki T, et al. The role of ICOS in the CXCR5+ follicular B helper T cell maintenance *in vivo*. *J Immunol.* (2005) 175:2340–8. doi: 10.4049/jimmunol.175.4.2340
50. Grimbacher B, Hutloff A, Schlesier M, Glocker E, Warnatz K, Drager R, et al. Homozygous loss of ICOS is associated with adult-onset common variable immunodeficiency. *Nat Immunol.* (2003) 4:261–8. doi: 10.1038/ni902
51. Ma CS, Wong N, Rao G, Avery DT, Torpy J, Hambridge T, et al. Monogenic mutations differentially affect the quantity and quality of T follicular helper cells in patients with human primary immunodeficiencies. *J Allergy Clin Immunol.* (2015) 136:993–1006 e1. doi: 10.1016/j.jaci.2015.05.036
52. Yu D, Tan AH, Hu X, Athanasopoulos V, Simpson N, Silva DG, et al. Roquin represses autoimmunity by limiting inducible T-cell co-stimulator messenger RNA. *Nature* (2007) 450:299–303. doi: 10.1038/nature06253
53. Vinuesa CG, Cook MC, Angelucci C, Athanasopoulos V, Rui L, Hill KM, et al. A RING-type ubiquitin ligase family member required to repress follicular helper T cells and autoimmunity. *Nature* (2005) 435:452–8. doi: 10.1038/nature03555
54. Choi YS, Kageyama R, Eto D, Escobar TC, Johnston RJ, Monticelli L, et al. ICOS receptor instructs T follicular helper cell versus effector cell differentiation via induction of the transcriptional repressor Bcl6. *Immunity* (2011) 34:932–46. doi: 10.1016/j.immuni.2011.03.023
55. Xu H, Li X, Liu D, Li J, Zhang X, Chen X, et al. Follicular T-helper cell recruitment governed by bystander B cells and ICOS-driven motility. *Nature* (2013) 496:523–7. doi: 10.1038/nature12058
56. Gigoux M, Shang J, Pak Y, Xu M, Choe J, Mak TW, et al. Inducible costimulator promotes helper T-cell differentiation through phosphoinositide 3-kinase. *Proc Natl Acad Sci USA* (2009) 106:20371–6. doi: 10.1073/pnas.0911573106
57. Stone EL, Pepper M, Katayama CD, Kerdiles YM, Lai CY, Emslie E, et al. ICOS coreceptor signaling inactivates the transcription factor FOXO1 to promote Tfh cell differentiation. *Immunity* (2015) 42:239–51. doi: 10.1016/j.immuni.2015.01.017
58. Xiao N, Eto D, Elly C, Peng G, Crotty S, Liu YC. The E3 ubiquitin ligase Itch is required for the differentiation of follicular helper T cells. *Nat Immunol.* (2014) 15: 657–66. doi: 10.1038/ni.2912
59. Kerdiles YM, Beisner DR, Tinoco R, Dejean AS, Castrillon DH, DePinho RA, et al. Foxo1 links homing and survival of naive T cells by regulating L-selectin, CCR7 and interleukin 7 receptor. *Nat Immunol.* (2009) 10: 176–84. doi: 10.1038/ni.1689
60. Lee JY, Skon CN, Lee YJ, Oh S, Taylor JJ, Malhotra D, et al. The transcription factor KLF2 restrains CD4(+) T follicular helper cell differentiation. *Immunity* (2015) 42:252–64. doi: 10.1016/j.immuni.2015.01.013
61. Parry RV, Rumbley CA, Vandenbergh LH, June CH, Riley JL. CD28 and inducible costimulatory protein Src homology 2 binding domains show distinct regulation of phosphatidylinositol 3-kinase, Bcl-xL, and IL-2 expression in primary human CD4 T lymphocytes. *J Immunol.* (2003) 171:166–74. doi: 10.4049/jimmunol.171.1.166
62. Arimura Y, Kato H, Dianzani U, Okamoto T, Kamekura S, Buonfiglio D, et al. A co-stimulatory molecule on activated T cells, H4/ICOS, delivers specific signals in T(h) cells and regulates their responses. *Int Immunol.* (2002) 14:555–66. doi: 10.1093/intimm/14.5.555
63. Ouyang W, Liao W, Luo CT, Yin N, Huse M, Kim MV, et al. Novel Foxo1-dependent transcriptional programs control T(reg) cell function. *Nature* (2012) 491:554–9. doi: 10.1038/nature11581
64. Rolf J, Fairfax K, Turner M. Signaling pathways in T follicular helper cells. *J Immunol.* (2010) 184:6563–8. doi: 10.4049/jimmunol.1000202
65. Haynes NM, Allen CD, Lesley R, Ansel KM, Killeen N, Cyster JG. Role of CXCR5 and CCR7 in follicular Th cell positioning and appearance of a programmed cell death gene-1-high germinal center-associated subpopulation. *J Immunol.* (2007) 179:5099–108. doi: 10.4049/jimmunol.179.8.5099
66. Chemnitz JM, Parry RV, Nichols KE, June CH, Riley JL. SHP-1 and SHP-2 associate with immunoreceptor tyrosine-based switch motif of programmed death 1 upon primary human T cell stimulation, but only receptor ligation prevents T cell activation. *J Immunol.* (2004) 173:945–54. doi: 10.4049/jimmunol.173.2.945

67. Hui E, Cheung J, Zhu J, Su X, Taylor MJ, Wallweber HA, et al. T cell costimulatory receptor CD28 is a primary target for PD-1-mediated inhibition. *Science* (2017) 355:1428–33. doi: 10.1126/science.aaf1292
68. Francisco LM, Salinas VH, Brown KE, Vanguri VK, Freeman GJ, Kuchroo VK, et al. PD-L1 regulates the development, maintenance, and function of induced regulatory T cells. *J Exp Med.* (2009) 206:3015–29. doi: 10.1084/jem.20090847
69. Shi J, Hou S, Fang Q, Liu X, Liu X, Qi H. PD-1 Controls follicular T helper cell positioning and function. *Immunity* (2018) 49:264–74.e4. doi: 10.1016/j.immuni.2018.06.012
70. Tian Y, Mollo SB, Harrington LE, Zajac AJ. IL-10 Regulates Memory T Cell Development and the balance between Th1 and follicular Th cell responses during an acute viral infection. *J Immunol.* (2016) 197:1308–21. doi: 10.4049/jimmunol.1502481
71. Migone TS, Rodig S, Cacalano NA, Berg M, Schreiber RD, Leonard WJ. Functional cooperation of the interleukin-2 receptor beta chain and Jak1 in phosphatidylinositol 3-kinase recruitment and phosphorylation. *Mol Cell Biol.* (1998) 18:6416–22. doi: 10.1128/MCB.18.11.6416
72. Truitt KE, Mills GB, Turck CW, Imboden JB. SH2-dependent association of phosphatidylinositol 3'-kinase 85-kDa regulatory subunit with the interleukin-2 receptor beta chain. *J Biol Chem.* (1994) 269:5937–43.
73. Gu H, Maeda H, Moon JJ, Lord JD, Yoakim M, Nelson BH, et al. New role for Shc in activation of the phosphatidylinositol 3-kinase/Akt pathway. *Mol Cell Biol.* (2000) 20:7109–20. doi: 10.1128/MCB.20.19.7109-7120.2000
74. Cornish GH, Sinclair LV, Cantrell DA. Differential regulation of T-cell growth by IL-2 and IL-15. *Blood* (2006) 108:600–8. doi: 10.1182/blood-2005-12-4827
75. Karnitz LM, Burns LA, Sutor SL, Blenis J, Abraham RT. Interleukin-2 triggers a novel phosphatidylinositol 3-kinase-dependent MEK activation pathway. *Mol Cell Biol.* (1995) 15:3049–57. doi: 10.1128/MCB.15.6.3049
76. Ross SH, Cantrell DA. Signaling and function of interleukin-2 in T lymphocytes. *Annu Rev Immunol.* (2018) 36:411–33. doi: 10.1146/annurev-immunol-042617-053352
77. Jacobs MD, Black J, Futer O, Swenson L, Hare B, Fleming M, et al. Pim-1 ligand-bound structures reveal the mechanism of serine/threonine kinase inhibition by LY294002. *J Biol Chem.* (2005) 280:13728–34. doi: 10.1074/jbc.M413155200
78. Pearce LR, Komander D, Alessi DR. The nuts and bolts of AGC protein kinases. *Nat Rev Mol Cell Biol.* (2010) 11:9–22. doi: 10.1038/nrm2822
79. Finlay DK, Rosenzweig E, Sinclair LV, Feijoo-Carnero C, Hukelmann JL, Rolf J, et al. PDK1 regulation of mTOR and hypoxia-inducible factor 1 integrate metabolism and migration of CD8+ T cells. *J Exp Med.* (2012) 209:2441–53. doi: 10.1084/jem.20112607
80. Johnston RJ, Choi YS, Diamond JA, Yang JA, Crotty S. STAT5 is a potent negative regulator of TFH cell differentiation. *J Exp Med.* (2012) 209:243–50. doi: 10.1084/jem.20111174
81. Ballesteros-Tato A, Leon B, Graf BA, Moquin A, Adams PS, Lund FE, et al. Interleukin-2 inhibits germinal center formation by limiting T follicular helper cell differentiation. *Immunity* (2012) 36:847–56. doi: 10.1016/j.immuni.2012.02.012
82. Oestreich KJ, Mohn SE, Weinmann AS. Molecular mechanisms that control the expression and activity of Bcl-6 in TH1 cells to regulate flexibility with a TFH-like gene profile. *Nat Immunol.* (2012) 13:405–11. doi: 10.1038/ni.2242
83. Ray JP, Staron MM, Shyer JA, Ho PC, Marshall HD, Gray SM, et al. The Interleukin-2-mTORc1 kinase axis defines the signaling, differentiation, and metabolism of T helper 1 and follicular B helper T cells. *Immunity* (2015) 43:690–702. doi: 10.1016/j.immuni.2015.08.017
84. Ye L, Lee J, Xu L, Mohammed AU, Li W, Hale JS, et al. mTOR Promotes Antiviral Humoral Immunity by Differentially Regulating CD4 Helper T Cell and B Cell Responses. *J Virol.* (2017) 91:e01653–16. doi: 10.1128/JVI.01653-16
85. Yang J, Lin X, Pan Y, Wang J, Chen P, Huang H, et al. Critical roles of mTOR Complex 1 and 2 for T follicular helper cell differentiation and germinal center responses. *Elife* (2016) 5:e17936. doi: 10.7554/eLife.17936
86. Macintyre AN, Finlay D, Preston G, Sinclair LV, Waugh CM, Tamas P, et al. Protein kinase B controls transcriptional programs that direct cytotoxic T cell fate but is dispensable for T cell metabolism. *Immunity* (2011) 34:224–36. doi: 10.1016/j.immuni.2011.01.012
87. Grzes KM, Swamy M, Hukelmann JL, Emslie E, Sinclair LV, Cantrell DA. Control of amino acid transport coordinates metabolic reprogramming in T-cell malignancy. *Leukemia* (2017) 31:2771–9. doi: 10.1038/leu.2017.160
88. Wu T, Shin HM, Moseman EA, Ji Y, Huang B, Harly C, et al. TCF1 Is Required for the T Follicular Helper Cell Response to Viral Infection. *Cell Rep.* (2015) 12:2099–110. doi: 10.1016/j.celrep.2015.08.049
89. Choi YS, Gullicksrud JA, Xing S, Zeng Z, Shan Q, Li F, et al. LEF-1 and TCF-1 orchestrate T(FH) differentiation by regulating differentiation circuits upstream of the transcriptional repressor Bcl6. *Nat Immunol.* (2015) 16:980–90. doi: 10.1038/ni.3226
90. Xu L, Cao Y, Xie Z, Huang Q, Bai Q, Yang X, et al. The transcription factor TCF-1 initiates the differentiation of T(FH) cells during acute viral infection. *Nat Immunol.* (2015) 16:991–9. doi: 10.1038/ni.3229
91. Yu Q, Sharma A, Oh SY, Moon HG, Hossain MZ, Salay TM, et al. T cell factor 1 initiates the T helper type 2 fate by inducing the transcription factor GATA-3 and repressing interferon-gamma. *Nat Immunol.* (2009) 10:992–9. doi: 10.1038/ni.1762
92. Lin WH, Adams WC, Nish SA, Chen YH, Yen B, Rothman NJ, et al. Asymmetric PI3K signaling driving developmental and regenerative cell fate bifurcation. *Cell Rep.* (2015) 13:2203–18. doi: 10.1016/j.celrep.2015.10.072
93. Nish SA, Zens KD, Kratchmarov R, Lin WW, Adams WC, Chen YH, et al. CD4+ T cell effector commitment coupled to self-renewal by asymmetric cell divisions. *J Exp Med.* (2017) 214:39–47. doi: 10.1084/jem.20161046
94. Kim MV, Ouyang W, Liao W, Zhang MQ, Li MO. The transcription factor Foxo1 controls central-memory CD8+ T cell responses to infection. *Immunity* (2013) 39:286–97. doi: 10.1016/j.immuni.2013.07.013
95. Wu T, Ji Y, Moseman EA, Xu HC, Mangani M, Kirby M, et al. The TCF1-Bcl6 axis counteracts type I interferon to repress exhaustion and maintain T cell stemness. *Sci Immunol.* (2016) 1:eaai8593. doi: 10.1126/sciimmunol.aai8593
96. Hess Michelini R, Doedens AL, Goldrath AW, Hedrick SM. Differentiation of CD8 memory T cells depends on Foxo1. *J Exp Med.* (2013) 210:1189–200. doi: 10.1084/jem.20130392
97. Jeannot G, Boudousquie C, Gardiol N, Kang J, Huelsken J, Held W. Essential role of the Wnt pathway effector Tcf-1 for the establishment of functional CD8 T cell memory. *Proc Natl Acad Sci USA.* (2010) 107:9777–82. doi: 10.1073/pnas.0914127107
98. Zhou X, Yu S, Zhao DM, Hartly JT, Badovinac VP, Xue HH. Differentiation and persistence of memory CD8(+) T cells depend on T cell factor 1. *Immunity* (2010) 33:229–40. doi: 10.1016/j.immuni.2010.08.002
99. Sayin I, Radtke AJ, Vella LA, Jin W, Wherry EJ, Buggert M, et al. Spatial distribution and function of T follicular regulatory cells in human lymph nodes. *J Exp Med.* (2018) 215: jem.20171940. doi: 10.1084/jem.20171940
100. Fu W, Liu X, Lin X, Feng H, Sun L, Li S, et al. Deficiency in T follicular regulatory cells promotes autoimmunity. *J Exp Med.* (2018) 215:815–25. doi: 10.1084/jem.20170901
101. Linterman MA, Pierson W, Lee SK, Kallies A, Kawamoto S, Rayner TF, et al. Foxp3+ follicular regulatory T cells control the germinal center response. *Nat Med.* (2011) 17:975–82. doi: 10.1038/nm.2425
102. Chung Y, Tanaka S, Chu F, Nurieva RI, Martinez GJ, Rawal S, et al. Follicular regulatory T cells expressing Foxp3 and Bcl-6 suppress germinal center reactions. *Nat Med.* (2011) 17:983–8. doi: 10.1038/nm.2426
103. Wollenberg I, Agua-Doce A, Hernandez A, Almeida C, Oliveira VG, Faro J, et al. Regulation of the germinal center reaction by Foxp3+ follicular regulatory T cells. *J Immunol.* (2011) 187:4553–60. doi: 10.4049/jimmunol.1101328
104. Sage PT, Sharpe AH. T follicular regulatory cells. *Immunol Rev.* (2016) 271:246–59. doi: 10.1111/imr.12411
105. Delgoffe GM, Kole TP, Zheng Y, Zarek PE, Matthews KL, Xiao B, et al. The mTOR kinase differentially regulates effector and regulatory T cell lineage commitment. *Immunity* (2009) 30:832–44. doi: 10.1016/j.immuni.2009.04.014
106. Haxhinasto S, Mathis D, Benoist C. The AKT-mTOR axis regulates *de novo* differentiation of CD4+Foxp3+ cells. *J Exp Med.* (2008) 205:565–74. doi: 10.1084/jem.20071477

107. Battaglia M, Stabilini A, Roncarolo MG. Rapamycin selectively expands CD4+CD25+FoxP3+ regulatory T cells. *Blood* (2005) 105:4743–8. doi: 10.1182/blood-2004-10-3932
108. Malek TR. The biology of interleukin-2. *Annu Rev Immunol.* (2008) 26:453–79. doi: 10.1146/annurev.immunol.26.021607.090357
109. Botta D, Fuller MJ, Marquez-Lago TT, Bachus H, Bradley JE, Weinmann AS, et al. Dynamic regulation of T follicular regulatory cell responses by interleukin 2 during influenza infection. *Nat Immunol.* (2017) 18:1249–60. doi: 10.1038/ni.3837
110. Xu L, Huang Q, Wang H, Hao Y, Bai Q, Hu J, et al. The Kinase mTORC1 promotes the generation and suppressive function of follicular regulatory T cells. *Immunity* (2017) 47:538–51 e5. doi: 10.1016/j.immuni.2017.08.011
111. Essig K, Hu D, Guimaraes JC, Alterauge D, Edelmann S, Raj T, et al. Roquin suppresses the PI3K-mTOR signaling pathway to inhibit T helper cell differentiation and conversion of treg to Tfr cells. *Immunity* (2017) 47:1067–1082 e12. doi: 10.1016/j.immuni.2017.11.00
112. Shekhar S, Yang X. The darker side of follicular helper T cells: from autoimmunity to immunodeficiency. *Cell Mol Immunol.* (2012) 9:380–5. doi: 10.1038/cmi.2012.26
113. Simpson N, Gatenby PA, Wilson A, Malik S, Fulcher DA, Tangye SG, et al. Expansion of circulating T cells resembling follicular helper T cells is a fixed phenotype that identifies a subset of severe systemic lupus erythematosus. *Arthritis Rheum.* (2010) 62:234–44. doi: 10.1002/art.25032
114. Jung S, Gamez-Diaz L, Proietti M, Grimbacher B. “Immune TOR-opathies,” a Novel Disease Entity in Clinical Immunology. *Front Immunol* (2018) 9: 966. doi: 10.3389/fimmu.2018.00966
115. Fruman DA, Bismuth G. Fine tuning the immune response with PI3K. *Immunol Rev.* (2009) 228:253–72. doi: 10.1111/j.1600-065X.2008.00750.x
116. Haylock-Jacobs S, Comerford I, Bunting M, Kara E, Townley S, Klingler-Hoffmann M, et al. PI3Kdelta drives the pathogenesis of experimental autoimmune encephalomyelitis by inhibiting effector T cell apoptosis and promoting Th17 differentiation. *J Autoimmun.* (2011) 36:278–87. doi: 10.1016/j.jaut.2011.02.006
117. Shrestha S, Yang K, Guy C, Vogel P, Neale G, Chi H. Treg cells require the phosphatase PTEN to restrain TH1 and TFH cell responses. *Nat Immunol.* (2015) 16:178–87. doi: 10.1038/ni.3076
118. Suzuki A, Kaisho T, Ohishi M, Tsukio-Yamaguchi M, Tsubata T, Koni PA, et al. Critical roles of Pten in B cell homeostasis and immunoglobulin class switch recombination. *J Exp Med.* (2003) 197:657–67. doi: 10.1084/jem.20021101
119. Durand CA, Hartvigsen K, Fogelstrand L, Kim S, Iritani S, Vanhaesebroeck B, et al. Phosphoinositide 3-kinase p110 delta regulates natural antibody production, marginal zone and B-1 B cell function, and autoantibody responses. *J Immunol.* (2009) 183:5673–84. doi: 10.4049/jimmunol.0900432
120. Jayachandran N, Mejia EM, Sheikholeslami K, Sher AA, Hou S, Hatch GM, et al. TAPP adaptors control B cell metabolism by modulating the phosphatidylinositol 3-kinase signaling pathway: a novel regulatory circuit preventing autoimmunity. *J Immunol.* (2018) 201:406–16. doi: 10.4049/jimmunol.1701440
121. Banham-Hall E, Clatworthy MR, Okkenhaug K. The therapeutic potential for pi3k inhibitors in autoimmune rheumatic diseases. *Open Rheumatol J.* (2012) 6:245–58. doi: 10.2174/1874312901206010245
122. Stark AK, Sriskantharajah S, Hessel EM, Okkenhaug K. PI3K inhibitors in inflammation, autoimmunity and cancer. *Curr Opin Pharmacol.* (2015) 23:82–91. doi: 10.1016/j.coph.2015.05.017
123. Zarate-Blades CR, Horai R, Caspi RR. Regulation of autoimmunity by the microbiome. *DNA Cell Biol.* (2016) 35:455–8. doi: 10.1089/dna.2016.3432
124. Rao VK, Webster S, Dalm V, Sediva A, van Hagen PM, Holland S, et al. Effective ‘Activated PI3Kdelta Syndrome’-targeted therapy with the PI3Kdelta inhibitor leniolisib. *Blood* (2017) 130:2307–16. doi: 10.1182/blood-2017-08-801191

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2019 Preite, Huang, Cannons, McGavern and Schwartzberg. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



Molecular Basis of the Differentiation and Function of Virus Specific Follicular Helper CD4⁺ T Cells

Qizhao Huang^{1,2}, Jianjun Hu², Jianfang Tang², Lifan Xu^{2*} and Lilin Ye^{2*}

¹ Cancer Center, The General Hospital of Western Theater Command, Chengdu, China, ² Institute of Immunology, Third Military Medical University, Chongqing, China

OPEN ACCESS

Edited by:

Maria Pia Cicalese,
San Raffaele Scientific Institute
(IRCCS), Italy

Reviewed by:

Francois Villinger,
University of Louisiana at Lafayette,
United States
Linrong Lu,
Zhejiang University, China

*Correspondence:

Lifan Xu
xlftofu@sina.com
Lilin Ye
yellinlcmv@tmmu.edu.cn

Specialty section:

This article was submitted to
T Cell Biology,
a section of the journal
Frontiers in Immunology

Received: 25 July 2018

Accepted: 29 January 2019

Published: 15 February 2019

Citation:

Huang Q, Hu J, Tang J, Xu L and Ye L
(2019) Molecular Basis of the
Differentiation and Function of Virus
Specific Follicular Helper CD4⁺ T
Cells. *Front. Immunol.* 10:249.
doi: 10.3389/fimmu.2019.00249

Keywords: follicular T helper cells, acute viral infection, chronic viral infection, differentiation, CD4 T cells +

INTRODUCTION

Based on the biological process, viral infections can be divided into two groups: acute viral infection and chronic viral infection. During acute infections, virus is thoroughly eliminated by the orchestration between both innate and adaptive immune cells; whereas, certain types of viruses can effectively evade immune system and persist at a certain level in the host for long time in chronic infections (1). Numbers of specific immune effector mechanisms, coordinating with non-specific defense mechanisms, prevent or eliminate most viral infections. In terms of adaptive immune cells, CD8⁺ T cell- and CD4⁺ T cell-mediated immune responses play a critical role in the control of viral infection. During acute viral infection, virus-specific CD8⁺ T cells differentiate into cytotoxic T lymphocytes (CTL) to efficiently eliminate virus-infected target cells and progressively transit into memory CD8⁺ T cells after viral eradication. Memory CD8⁺ T cells are maintained for a long time in the absence of antigen and can exert rapid effector functions in response to previously encountered antigens.

After a transit time in the blood, the majority of mature naïve CD4⁺ T cells produced by the thymus migrate to secondary lymphoid tissues, continually patrolling, and browsing for antigens they can recognize. After entering a lymph node, T cells scan the processed peptide-MHC complexes on the surface of DCs in the paracortex or T-cell zone. DCs that have processed antigen at the sites of infection arrive in the paracortex soon after infection. Upon viral infection, virus-specific CD4⁺ T cells mainly differentiate into Th1 and Tfh (follicular helper T cell) cells, but not other helper subsets, such as Th2, Th17, and Th9 due to the strong type-I inflammation. And the divergence of Tfh and Th1 differentiation fates begin immediately after activation and are faithfully maintained through the life cycle (2). Through interactions between S1P1 receptors and S1P, the Th1 subset leaves the lymph node and travel to sites of infection. And they predominantly function through secreting IL-2, IFN- γ and TNF α and are responsible for many typical cell-mediated effects, including activation of CTL and macrophages. In contrast, virus-specific Tfh cells, characterized by

high expression of chemokine receptor CXCR5, are endowed with the ability of migrating into B cell follicles in response to chemokine CXCL13 (3, 4), where they facilitate the maturation of GC B cells by interacting with cognate virus-specific B cells and providing “help” signals such as interleukin 21 (IL-21), IL-4, CD40L and inducible costimulatory molecules (ICOS) (5).

Tfh differentiation is generally characterized as a multistage, multifactorial process (**Figure 1**) (6). Upon recognition of virus peptide-MHC complex (p-MHC) presented by dendritic cells (DCs), CD4⁺ T cells adopting Tfh fate upregulate the “master regulator” Bcl-6 (7–9) within 2 or 3 days (10, 11). After engagement with DCs, Tfh cells move to the T-B border by upregulating CXCR5 and down-regulating CCR7 (10, 12). Here, they interact with cognate B cells and get sufficient signals that further support them migrating into B cell follicles and initiating GC reactions (13). During this process, the expression of Bcl-6 is enhanced, propelling the maturation of fully functional Tfh cells (14). Contrarily, Blimp1 (B lymphocyte-induced maturation protein-1), mainly expressed by non-Tfh effector cells, inhibits the expression of Bcl-6 and negatively regulates Tfh cell differentiation (7). Although the majority of Tfh cells originated from precursors in lymphoid tissues, several groups confirmed the existence of circulating CXCR5⁺ CD4⁺ T cells in mice or humans with ongoing immune responses, which were termed as peripheral Tfh (pTfh) (15–19). For instance, He et al. (18) demonstrated that pTfh consist of two parts: “effector” pTfh and “resting” pTfh cells, identified as CCR7^{lo}PD-1^{hi} and CCR7^{hi}PD-1^{lo}, respectively. They found that CCR7^{lo}PD-1^{hi}CXCR5⁺ CD4⁺ T cells express large amounts of IL-21, a key cytokine secreted by Tfh cells to support GC responses. And this population is able to further differentiate into mature Tfh cells and initiate GC formation. They believed that CCR7^{lo}PD-1^{hi} Tfh precursor cells can circulate to non-draining secondary lymphoid organs and rapidly differentiate into mature Tfh cells to support fast GC formation upon antigen reencounter. However, the underlying mechanisms of the ontology and differentiation of this population remain unsolved.

Tfh cells are essential for antibody-mediated humoral immunity against various pathogens. This review primarily focuses on the current understanding of the fate commitment, functional maturation, and memory formation of Tfh cells during acute viral infection. Moreover, we also focus on the role of Tfh cells during chronic viral infection, especially in HIV infection. Finally, we discuss the potential in boosting viral-specific Tfh cells for improving efficacies of anti-viral vaccines.

THE FATE COMMITMENT OF VIRUS-SPECIFIC Tfh CELLS VS. Th1 CELLS

The fine-tuned cooperation of cognate p-MHCII molecular interactions, co-stimulation, together with polarizing cytokine signals initiate the differentiation of functionally divergent CD4⁺ T helper (Th) cell subsets from their precursors (20). Of note, during acute viral infection, commitment to the Tfh lineage vs. Th1 lineage emerges as early as 24 to 48 h after infection. The

dichotomous commitment of Tfh cells vs. Th1 cells is largely linked to reciprocal regulation between key transcription factors Bcl6 and T-bet, and Bcl6 and Blimp-1 (21, 22).

At the priming stage, DCs regulate Tfh cell differentiation by controlling Clec9A expression, which facilitates the formation of a long-term immune synapse between DCs and T cells to promote Tfh differentiation (23, 24). Indeed, published work (25–27) has found that 24 h after T cell activation, T cells carrying high affinity TCRs can form long dynamic immune synapses with DC and are more inclined to differentiate into Tfh but not Th1 cells. In addition to interactions between membrane proteins of APC and Tfh precursors, secreted cytokines interleukin-6 (IL-6) and IL-21 also contribute to Tfh differentiation. Several groups confirmed that IL-6 and IL-21 signaling via the transcription factor STAT3 enhances the upregulated expression of Bcl6, which is the master regulator of Tfh differentiation. Nonetheless, IL-2 suppresses Tfh fates by activating STAT5 and restricting STAT3 binding to the *Bcl6* locus and also by promoting the expression of Blimp-1, which divert differentiation away from the Tfh pathway (20, 28–31). Propelled by the antagonism of Bcl6 and Blimp-1, activated CD4⁺ T cells undergo a bimodal fate decision during acute viral infection: becoming either Tfh (Bcl6⁺Blimp1[−]) cells or Th1 (Bcl6[−]Blimp1⁺) cells. Notably, the transcription factor TCF-1 (t cell factor 1, coded by gene *Tcf-7*) has been confirmed to promote the early fate commitment to the Tfh lineage over Th1 lineage during acute viral infection (32–34). Using the LCMV-Armstrong and influenza virus infection model, we (32) found that the expression level of TCF-1 was significantly enhanced in Tfh cells while greatly diminished in Th1 cells. And such divergent expression mode occurred as early as 2 days post infection. TCF-1 potentially induced the expression of Bcl-6 but suppressed Blimp1 concomitantly, by directly binding to the *Bcl6* promoter region and *Prdm1* 5' regulatory region, respectively. Accordingly, virus-specific CD4⁺ T cells deficient in TCF-1 expression almost failed in Tfh differentiation. Notably, TCF-1 seems to specifically regulate Tfh cell differentiation in the context of viral infection, but dispensable for regulating Tfh differentiation during protein immunization (32, 33).

Apart from the master regulator Bcl-6, a network of several other transcription factors also participates in controlling the differentiation of Tfh cells during acute viral infection. For example, it has been confirmed that through two different but complementary mechanisms, the transcription factor KLF2 (Krüppel-like factor 2) functions to restrain Tfh cell generation. Lee et al. (35) found that KLF2 promotes the expression of the trafficking receptor S1PR1, the downregulation of which is essential for efficient Tfh cell differentiation. On the other hand, KLF2 favors the expression of several transcription factors that inhibit Tfh differentiation, such as Blimp1, Tbet, and GATA3. And KLF2 was also reported to suppress the transcription of *Cxcr5* by directly binding to its genomic region (36). Importantly, although Tbet is the master transcriptional regulator of Th1 cells, which were thought to inhibit Tfh cell differentiation, Tfh cells do exhibit medium to high levels of Tbet expression in the LCMV infection model (2). Recently, it has been reported that T-bet is virtually essential for the optimal expansion, proliferation, and maintenance of Tfh cells during acute viral

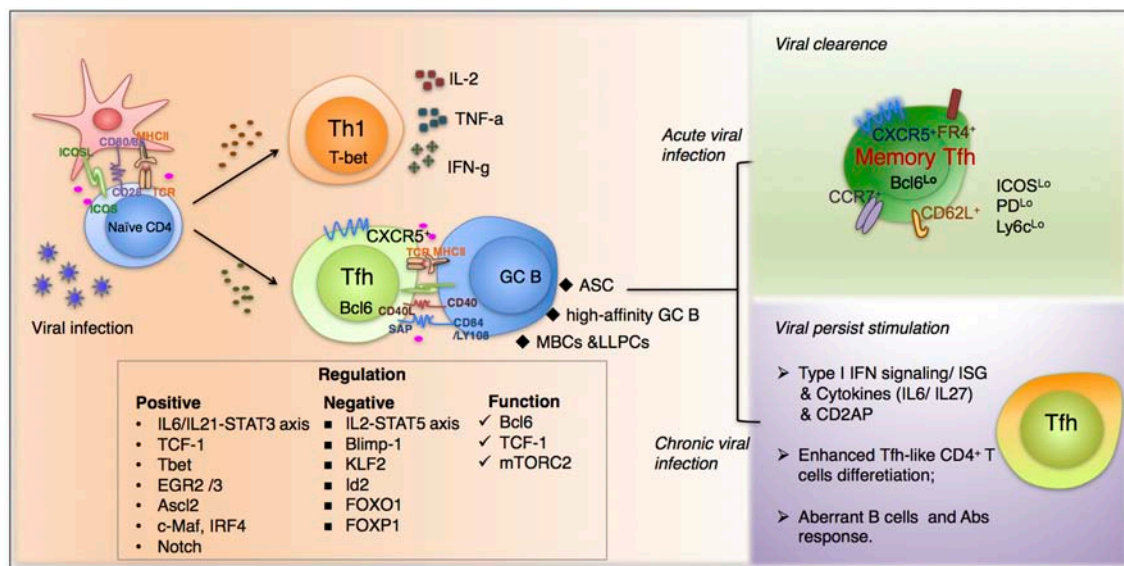


FIGURE 1 | The fate commitment of Tfh cells during viral infection. Upon viral infection, virus-specific CD4⁺T cells mainly differentiate into Th1 and Tfh cells. Th1 subset predominantly function through secreting IL-2, IFN- γ , and TNF α and is responsible for many typical cell-mediated effects. In contrast, virus-specific Tfh cells, characterized by high expression of chemokine receptor CXCR5, are endowed with the ability of migrating into B cell follicles in response to chemokine CXCL13. The fate commitment and function of Tfh cells require fine-tuned cooperation of cognate p-MHCII molecular interactions, co-stimulation, together with polarizing cytokine signals and other factors. Moreover, upon viral clearance, a proportion of Tfh cells will differentiation in memory cells, which are more active when encountered with the same pathogens. Additionally, during chronic viral infections, Tfh cells accumulate gradually and exhibit a distinguished transcriptional profile compared with that in acute infections. And many factors participate in the unique differentiation pattern during persistent infection, including type I interferon signaling, cytokines from IL-6 family et al.

infection (37). Besides, Fang et al. (38) demonstrated that at the early stage of CD4⁺ T cells response, the short-term expression of Tbet is critical for IFN- γ production in Th1-like Tfh cell subset. Additionally, transcription factors of the E-protein and Id families are well-appreciated for their role in T cell development. Shaw et al. (39) found that Tfh cells exhibited lower expression of Id2 than that of Th1 cells during acute viral infection and knockdown of Id2 via shRNA increased the frequency of Tfh cells. Furthermore, Th1 differentiation was significantly blocked by the deficiency of gene *Id2* during viral infection. Ogbe et al. (40) found that EGR2 (early growth response gene 2) and EGR3 play a vital role in directing the expression of *Bcl6* in Tfh cells. The differentiation of Tfh cells was impaired in *Egr2* and *Egr3* deficient mice post viral infection because of the defective expression of Bcl-6, resulting in a defective GC reaction and antibody production. Moreover, the overexpression of Bcl-6 in EGR2/3- deficient CD4⁺ T cells partially rescued the differentiation of Tfh cells and GC formation. Liu et al. (41) found that during influenza virus infection, the deletion of *Ascl2* in T cells results in impaired Tfh-cell development and germinal center response. Besides, in protein immunization or other infection models, several other TFs have been confirmed to participate in the regulation of the fate commitment of Tfh cells. For example, c-Maf, IRF4, and Notch signaling pathway has been confirmed to promote Tfh differentiation while FOXO1 and FOXP1 inhibit Tfh fate commitment (21, 42–47). Besides networks mediated by transcriptional factors, other different

signaling pathways also control the differentiation and function of Tfh cells. Tfh cell differentiation are closely associated with mTOR-mediated signaling pathways, which exert its effect by sensing and integrating environmental cues. During acute viral infection, the interleukin-2 (IL-2)-mTORC1 signaling axis orchestrates the reciprocal balance between Th1 and Tfh cell fates by promoting Th1 while inhibiting Tfh cell differentiation (20). In contrast, it is reported that mTORC2 was essential for Tfh cell differentiation (48, 49); specifically, mTORC2 mainly functions in the late stage of Tfh differentiation, promoting a Tfh transcriptional program and migratory ability toward B cell follicles (50).

Currently, however, our knowledge about Tfh cells is mainly derived from mouse models, although the gene expression pattern of mouse Tfh cells shares a high percentage of similarities with human Tfh, certain differences do exist between the two species. For instance, in mouse models, the ligand for CXCR5, CXCL13 is mainly expressed by stromal cells but not Tfh cells (6, 51). In humans, however, CXCL13 is primarily generated by Tfh cells, which may promote recruiting GC B cells to the light zone, where most Tfh cells and FDCs reside (52–54). Hence, further research is required for carefully profiling the differences between human and murine Tfh cells, which is critical for translating findings between the two species. Taken together, like Tfh cells developed in other scenarios, the fate commitment of virus-specific Tfh cells also follow a pathway involved a multistep and multifactorial process. Although we have gained a relatively

detailed understanding of the ontology and differentiation of Tfh cells during viral infection, there are still important gaps in our knowledge of the Tfh cell differentiation and underlying mechanisms during viral infection both in mouse and humans. In particular, how extrinsic stimuli from APCs and intrinsic factors, such as epigenetic modifications, regulate anti-viral Tfh differentiation await further investigations.

THE FUNCTIONAL MATURATION AND MAINTENANCE OF VIRUS-SPECIFIC Tfh CELLS

Currently, most licensed anti-viral vaccines protect vaccinated population by inducing long-lived neutralizing antibodies (55). Tfh cells play a critical role in helping B cells to differentiate into neutralizing-antibody-secreting plasma cells. Therefore, it is essential to understand the mechanisms by which Tfh cells co-opt B cell responses during viral infection, so as to effectively facilitate developing a novel vaccine or further improving the efficacies of licensed vaccines. Previous reviews have systematically summarized the functions of Tfh cells (6, 56), we herein mainly focus on the roles of these cells in the case of viral infection. After viral infection, SLAM-associated protein (SAP) expressed by Tfh cells is critical for the formation of germinal centers (57, 58), where Tfh cells facilitate the generation of long-lived memory B cells and plasma cells that produce virus-specific antibodies (57, 59). For example, CD4⁺ T cells have been confirmed to be essential for the generation of optimal antibody responses during infections with yellow fever virus (60), vaccinia virus (61), coronavirus (62), or vesicular stomatitis virus (VSV) (63). Collectively, Tfh cells play an important role in protective immunity to most, if not all, viruses.

In antiviral humoral immunity, Tfh cells help B cell activation and antibody production in the form of receptor ligand interactions and cytokine signaling. Firstly, Tfh cells highly express CD40 ligand (CD40L), whose interaction with CD40 expressed on B cells is vital to multiple stages and aspects of B cell response. Using LCMV, Pichinde virus, and VSV infection model, Borrow et al. (64) found that CD40L-deficient mice exhibit severely compromised humoral immune responses, supported by low antiviral antibody production, absence of germinal center and memory B cell formation. Consistently, CD40L/CD40 was also reported to be important for generating optimal humoral responses against HSV and influenza virus (65, 66). During LCMV, VSV, and influenza virus infection model, the expression of ICOS (inducible T cell co-stimulator) by Tfh cells has also been reported to be crucial for germinal center formation (6) and optimal induction of humoral responses (67). Other co-stimulatory molecules that promote the T-B conjugates, including SAP and SLAM family are also required for Tfh differentiation as well as Tfh function (6). It is important to appreciate that both defective Tfh cell number and damaged Tfh function can lead to impaired GC response. To more precisely evaluate the effector function of already differentiated Tfh cells during viral infection *in vivo*, our group combines *ERT2^{cre}* conditional knockout mice with mature Tfh cells adoptive

transfer strategy to determine their “help” ability in promoting the formation of GC and plasma cells (32, 50). The expression of these B-cell helping molecules (CD40L and ICOS) in Tfh cells appears to be coordinated by Bcl-6 (68, 69), TCF-1 (32), and mTORC2 (50). Further studies are needed to determine the importance of additional molecular signals between Tfh cells and B cells in the production of protective antibody responses during viral infection.

THE MEMORY FORMATION OF VIRUS-SPECIFIC Tfh CELLS

Most of the virus-specific effector CD4⁺ T cells will die and only a small portion of them will survive and further differentiate into memory T cells after the elimination of a viral infection. The features of memory lymphocyte generally includes (1) antigen experienced (these cells have undergone antigen-driven expansion); (2) can survive for a long time (undergo homeostatic proliferation) in the absence of antigenic stimulation; (3) self-renewable by homeostatic proliferation; (4) rapidly recall their effector functions in response to re-challenge (70). Memory CD4⁺ T cells respond much faster than naive T cells, require less synergistic stimulation to respond to low antigen doses, and are more active when challenged by pathogens (71). Recent studies suggest both effector Tfh and Th1 cells can differentiate into memory cells.

Series of studies have clearly demonstrated the existence of memory Tfh cell in both mice and human (Figure 1) (2, 17, 72–75). These studies provide important insights into the characteristics of Tfh cells that can differentiate into long-lived memory-type cells which are endowed with capacity to reboost Tfh-specific effector functions when encountered with the same antigen (76). Meanwhile, considering the long persistence of GC reactions and antigen retention by FDC, it is important to appreciate that GC Tfh cells are not confined to one single GC. Once GC Tfh cells have differentiated and provided help to GC B cells, they can continually enter a different GC or exit GC and emigrate to neighboring follicles (77, 78), where no antigen was presented and Tfh cells acquires a less activated, less polarized phenotype. In this situation, by downregulating Bcl6 expression and upregulating IL-7Ra, Tfh cells gradually transit into a resting memory state (73, 78). In acute viral infection for instance, after the clearance of LCMV in mice, virus-specific CD4⁺ T cells that survived the contraction phase can be maintained for 60–150 days (2). Among these cells, CXCR5⁺Ly6c^{lo} resting CD4⁺ T cells shared similarities with effector Tfh cells, both phenotypically and transcriptionally, and can rapidly recall a secondary wave of effector Tfh response even in the absence of B cells, supporting that CXCR5⁺ memory cells have been imprinted with a Tfh-biased cell program (2). Cell markers that can clearly define memory Tfh cells including high expression of CXCR5, FR4 (79), CCR7, CD62L, and low expression of Bcl-6, ICOS, PD-1, and Ly6c (80). Currently, the differentiation pattern of memory Tfh cell remains controversial. An important question is whether the fate of memory Tfh cells is determined before or after effector phase. Meanwhile, given that Th1 vs. Tfh differentiation are

regulated by strength and/or duration of TCR signaling (81, 82), which also influences memory CD4⁺ T cell differentiation (83), it is possible that Tfh effector population with different TCR strength varies in degrees of lineage commitment to CXCR5⁺ Tfh memory cells.

Besides, previous study indicated that memory Tfh cells are superior to naive T cells in helping B cells, and they promote faster B cell proliferation, higher antibody production and earlier class-switching reactions than naive CD4⁺ T cells (76). In many cases, the invasive virus can be quickly recognized and eradicated by pre-existing antibodies. But for viruses that bear high mutation rate (such as influenza virus and HIV), the most important point lies on faster antibody production, as the timely and efficient production of neutralizing antibodies targeting against new variants that escape from previously produced antibodies will be of great significance. Memory Tfh cells maintain a substantial level of CD40L (84) and are retained in the draining lymph nodes for more than 6 months (85). Besides, during several types of viral infections, such as Ebola virus (86), WNV (87), and influenza virus (88), memory Tfh cells can generate higher levels of cytokines as compared with those of naive T cells, which are likely to induce a more-potent B cell responses and to dictate the isotype of antibodies, which play key roles in the antibody responses specific for aforementioned viruses. Nonetheless, we currently know much less as to the molecular mechanisms underlying memory Tfh differentiation than those discovered with effector Tfh cells.

THE DIFFERENTIATION OF VIRUS-SPECIFIC Tfh CELLS DURING CHRONIC VIRAL INFECTION

We know far less about how chronic viral infections affect CD4⁺ T cell responses than we do about CD8⁺ T cell exhaustion. However, increasing attention has been paid to the impact of persistent viral infection on the function of CD4⁺ T cells and the importance of CD4⁺ T cells in chronic viral infection. Compared with acute viral infection, virus-specific CD4⁺ T cells in LCMV clone 13 persistent infection model exhibit deficiency in production of Th1-type effector cytokines and fail to function optimally following viral re-challenge (89). The loss of CD4⁺ T cell's ability to respond to persistent antigens may be due to high levels of antigens at the priming stage (90) and appears not to be regulated by the changes in APCs caused by chronic viral pathogens (89).

Similar to CD8⁺ T cell exhaustion in chronic infection, the virus-specific CD4⁺ T cell response has been altered profoundly as infection persists. The most significant phenotype of CD4⁺ T cell response during chronic viral infection is a defect in Th1, while increasing in Tfh response (**Figure 1**). And both in mouse and human chronic viral infections, the frequency of CXCR5⁺CD4⁺ T cells in spleen accumulates gradually, reaching approximately 60~70% of the viral-specific CD4⁺ T cells by day 30, whereas which were relatively lower at a frequency of 40~50% during Arm infection (91). The increased Tfh differentiation was accompanied by a loss in Th1, including decreased proliferative

potential and cytokine production (89). Thereby, to some extent, the immune system promotes antibody responses, which bear less immune-pathological risk compared to cytotoxic and pro-inflammatory T cell responses. Moreover, transcriptional profiling of viral-specific CD4⁺ T cells in LCMV clone 13 infection identified a loss in Th1 transcriptional signatures, as well as an enrichment of Tfh-associated transcripts (92). Upregulated CXCR5, Bcl-6, ICOS, OX40, and IL-21 expression suggest an enhanced Tfh-like CD4⁺ T cells phenotype (91). Very importantly, these additional Tfh-like CD4⁺ T cells are proven to have the ability to help B cells through *in vitro* culture, suggesting that they are equipped with some key signatures of conceptual Tfh cells and remain suboptimal functions, such as the ability to facilitate coordinate B cell response and production of antibody (91, 93). Although we have not yet got a comprehensive understanding of the biased differentiation of Tfh cells in chronic viral infection, previous research proved that type I IFN signaling may be an important mediator involved in the shift from Th1 to Tfh cells (94–97). Several groups also demonstrated the skewed differentiation toward Tfh cells during chronic LCMV infection is firmly related to cytokines (such as IL-6/ IL-27) signaling through the IL-6 family receptor pathway (98, 99). Recently, Raju et al. (100) found that the deficiency of the signaling adaptor CD2AP (CD2-associated protein) promotes CD4⁺ T cell differentiation toward Tfh lineage during chronic LCMV infection, leading to better control of viral infection by enhanced GC response. They demonstrated that the strengthened Tfh differentiation is associated with extended duration of TCR signaling and enhanced cytokine production of CD2AP-deficient CD4⁺ T cells specifically under Th1 conditions. To be noted, the increased CXCR5 level may also contributed by another CD4⁺ T cell subpopulation, Tfr cells (101, 102), coincide with upregulated Foxp3 expression during chronic infection (103). Although the function of Tfr cells is incompletely understood, especially in chronic infection, the increased Tfr differentiation suggest an active follicular program in chronic infection. And this follicular program may be not only confined to CD4⁺ T cell lineage, confirmed by newly identified CXCR5⁺CD8⁺ T cells described both in mice and human chronic viral infection (104–106).

Although viral persistence redirects a shift in Tfh differentiation, it is not clear to what extent the function of Tfh cells generated during chronic viral infection gets changed. IL-21, canonical Tfh cytokine important for CD8⁺ T cell function in chronic viral infection (107–110), is increased within Tfh population. However, for humoral immunity, it seems that B cell could not get optimal help from increased Tfh in chronic LCMV infection. Firstly, the generation of neutralizing antibodies are impaired and delayed, whereas, non-neutralizing antibodies to LCMV increased considerably (111). Secondly, persisting viral infections can lead to polyclonal hypergammaglobulinemia and antibody-mediated autoimmunity, results of non-specific B cell activation by Tfh cells (112, 113). The delayed production of neutralizing antibodies and high production of poor quality antibodies indicate that the interaction between Tfh and B cells in chronic LCMV infection is dysregulated, leading to a suboptimal ability of Tfh to help B cells producing high-affinity antibodies.

As has been observed in LCMV, HIV, and SIV infections also have been reported to have increased frequency of CXCR5⁺CD4⁺ T cells (114, 115). Besides, compared with uninfected healthy donors, the transcription characteristics of Tfh cells in the SIV infection model were changed. It has been confirmed that the transcriptional signature of Tfh cells derived from SIV-infection models gets remarkably altered compared to those from healthy donors (116). The underlying mechanisms initiating and promoting a Tfh-like program in HIV infection still remain unsolved and the relationship between HIV infection and Tfh differentiation is complicated. During HIV or SIV infections, Tfh cells seem to act in a bilateral manner, both immunological and immunopathological: Firstly, Tfh cells are appreciated as an important cellular reservoir of replication-competent HIV virus, contributed by its special phenotype and follicular localization. It is demonstrated that Tfh cells located in B-cell follicles are preferentially targeted by HIV virus to form both long-term latent infection and the enduring generation of virulent particles (110, 117), and the anatomical separation of latently infected Tfh cells might represent a major barrier for HIV-specific CD8⁺ T cells, which are normally excluded from B-cell follicles, to effectively eradicate HIV infection (118–121). Secondly, Tfh is closely involved in developing antibody-based vaccines for HIV-1 infection, because functional Tfh-B cell interactions are key to production of effective antibodies in vaccination (16, 122). Consistent with mouse chronic infection models, Tfh cells do not provide adequate help to B cells even though these cells are expanded in HIV-infected individuals (increased Tfh frequency does not result in better B cell response). Instead, similar to mouse chronic infection of LCMV, abnormal B cell activation and hyper-gammaglobulinemia were observed in HIV-1 infection (112, 115, 123), which suggests the dysregulation of Tfh cell-mediated B cell help and disturbed Tfh-B cell interactions. Specifically, data from mass cytometry combined with TCR sequencing confirmed that compared with healthy individuals, Tfh cells in the lymph nodes of HIV⁺ individuals secreted interleukin-21 but were functionally and clonally restricted and this correlated with impaired isotype switching of B cells in the lymph nodes (124). Given the close relationship with Tfh from lymphoid tissues, circulating or peripheral Tfh cells have also been confirmed to be critical in HIV infection (16, 22, 125). He et al. (18) demonstrated that circulating CXCR5⁺CD4⁺ T cells are generated in a SAP independent manner (before they migrate to GC), and CCR7^{lo}PD-1^{hi} subset correlated with Tfh cell activity, providing a biomarker to monitor protective humoral immune responses during infection or vaccination. In a related study, combining cytokine production, functional properties as well as gene expression profile, Locci et al. (17) identified pTfh cells related to germinal center Tfh cells as resting CD45RO⁺PD-1⁺CXCR5⁺CXCR3⁻CD4⁺ T cells. And they confirmed that the frequency of this population positively correlates with the titers of HIV-specific broadly neutralizing antibodies in a large cohort of HIV-infected patients. Schultz et al. (15) found that during HIV infection, peripheral IL-21⁺CD4⁺ T cells show similarities with lymphoid tissue-resident Tfh cells phenotypically, transcriptionally, and functionally. And they also found that the numbers of HIV-specific IL-21-expressing

pTfh cell increased and their number positively correlated with antibody production in the ALVAC priming, AIDSVAX boosting immunization strategy used in the RV144 trial (the only HIV vaccine to demonstrate some signs of efficacy among human patients) when compared with the non-protective DNA prime-Ad5 boosting vaccine trial. Given that the timely development of high-affinity antibodies is central to the prevention and eradication of viral infection (126), further work is needed to understand the detailed mechanism underlying Tfh dysfunction during persistent viral infections.

The key feature of CD8⁺ T cell exhaustion is upregulated expression of co-inhibitory receptors, such as PD-1, Tim3, 2B4. Although CD4⁺ T cells sustained the expression of a set of co-inhibitors (127), however, the specific inhibitory receptors upregulated and the degree of expression between CD4⁺ and CD8⁺ T cells differed remarkably (92, 127). For example, the expression of 2B4 is biased toward exhausted CD8⁺ T cells, while PD-1, CTLA4 are preferentially expressed in CD4⁺ T cells, particularly in Tfh cells (92). Several groups (128) found that functionally impaired CD4⁺ T cells derived from HIV patients exhibit significant enhancement in proliferative potential after treatment targeting on CTLA-4 (129, 130), TIM3 (131) or PD-1 signaling blockade (132) *in vitro*. And the effector function of CD8⁺ T cells can be rescued through enhancement of CD4⁺ T cell response during chronic infection with LCMV (133, 134). These findings and others (135, 136) shed new lights on the design of vaccines against chronic viral infections. For Tfh cells, physiologically, PD-1 is assigned to provide inhibitory signals to GC Tfh cells, preventing excess cell proliferation during GC reaction (21). Good-Jacobson et al. (137) demonstrated that upon immunization, the deficiency of PD-1 or PD-1 ligands (PD-L1/PD-L2) results in higher frequency of Tfh cells. Whereas, they also found that the quality of Tfh cells is dramatically impaired by diminishing their capacity to synthesize important cytokines (such as IL-4/IL-21) while not promoting the development of an alternatively polarized T cell type. These results suggest a complex but critical role of PD-1 in Tfh cells. However, despite the above findings, the role of PD-1 in Tfh cells during chronic viral infection remains unclear. Whether the expression of PD-1 on Tfh cells equals exhaustion or whether this is part of their normal regulation and functional differentiation during persistent infection have not yet been fully discovered.

Most of the aforementioned knowledge about CD4⁺ T cell response during chronic viral infection has been obtained from studies in which animals are infected with a single virus. While valuable for identification of basic principles, this is not reflective of human biology, since human beings undergo repeated viral infections throughout their life span, most notably, multiple herpesviruses. The γ -herpesviruses (Gammaherpesviruses), including EBV (Epstein-Barr virus) and KSHV (Kaposi's sarcoma-associated herpesvirus), are associated with lymphoproliferative diseases and lymphomas and with the majority establishing latency in B lymphocytes (138). In mouse models, intranasal infection of mice with the murine γ -herpesvirus (MHV-68, shares biological and genetic homology with EBV) results in an acute lytic infection in the lung, followed by the establishment of lifelong latency in memory B cells,

dendritic cells, and macrophages (104, 139–141). Barton et al. (142) demonstrated that both the proportion and total number of $\text{IFN}\gamma^+$, $\text{TNF}\alpha^+$, and IL-2^+ CD4^+ T cells was increased in mice infected with MHV68 followed with LCMV-Armstrong re-challenge compared to that in mice solely infected with LCMV on day 8 post infection. This result reminded us that MHV68 latency may provide micro-environment in which effector CD4^+ T cell responses get enhanced during subsequent infection. Another study confirmed that signals from Tfh cell is critical for B cell latency during MHV68 infection. They found that the absence of these signals lead to a significant reduction in the number of MHV68 latently infected B cells (143). However, whether Tfh cells are selectively up-regulated during MHV68 chronic infection has not yet been fully illustrated. Apart from its fundamental role in supporting B cell latency in MHV68 infection, CD4^+ T cells may also control MHV68 replication in a CD8^+ T cell dependent or independent manner (138, 144). Recently, several groups (104, 145) identified a specialized group of cytotoxic T cells that expressed high level of the chemokine receptor CXCR5 (Tfc, Follicular cytotoxic T cells), which selectively entered B cell follicles and eradicated infected Tfh cells and B cells during HIV/SIV or EBV infection, respectively. Given that Tfh and Tfc cells have a similar histological location, it will be of interests to determine whether these two subsets have interaction or crosstalk during chronic viral infection.

Collectively speaking, further dissection of unique molecular mechanisms underlying differentiation and functionality of Tfh cells in chronic viral infection will provide opportunities for harnessing this population to prevent and treat chronic viral infection.

PERSPECTIVE

Currently, the transcriptional regulation of the ontogeny and development of Tfh cell has been extensively investigated. However, the field just starts to dissect the complexities of cellular metabolism within Tfh cell as well as its epigenetic signatures, particularly, in the scenario of viral infections. It is well-acknowledged that the differentiation of Tfh cells is accompanied by unique metabolic alterations required to meet their cellular bioenergetic demands. During acute viral infection, Tfh cells exhibited a relatively quiescent metabolic state when compared to Th1 lineage, characterized by reduced glucose uptake and mitochondrial respiration, as well as lowered maximal respiratory capacity and extracellular acidification. However, despite Tfh cells showing reduced metabolic capacity, they still require glycolysis as well as oxidative phosphorylation to provide sufficient energy and substrates for their specific function (20). Zeng et al. (49) found that mTOR, combining metabolic signals and transcriptional activity, plays as a central control station in Tfh differentiation. Activated by costimulatory molecule ICOS, mTOR acts to drive glycolysis and lipogenesis and subsequently promotes Tfh cell responses during acute viral infection. Given that GC-Tfh cells have a different localization

compared to outside Tfh cells. They may have distinct metabolic features influenced by unique cellular and nutritional contact within each microenvironment. Moreover, it is possible that memory Tfh cells differs from effector Tfh cells in metabolism as described in effector vs. memory CD8^+ T cells. Whether and how cellular metabolism influence the formation of memory Tfh cells still need further investigation.

Besides metabolic issues, the differentiation of Tfh cells as well as other CD4^+ T helper (Th) cells are firmly correlated with specific epigenetic modifications (146, 147). By generating T cell-specific UTX (ubiquitously transcribed tetratricopeptide repeat, X chromosome) deficient mice, Cook et al. (148) found that during chronic but not acute, virus infection, Tfh differentiation were significantly impaired in UTX deficient mice, which in turn leads to suboptimal formation of germinal center and production of virus-specific IgG. Mechanistically, the absence of UTX leads to the upregulation of H3K27 methylation which further results in decreased expression of *IL-6R* alpha and other Tfh lineage-related genes. Nishizawa et al. (149) demonstrated that Bcl-6 is highly expressed in angioimmunoblastic T-cell lymphoma (AITL) and peripheral T-cell lymphomas (PTCL) containing tumor cells with Tfh features. In their research, hypermethylation of the *Bcl6* locus followed by Bcl-6 upregulation, combined with *TET2* mutations, was thought to be the key event for lymphoma development which may result in biased Tfh differentiation and eventually contribute to AITL/PTCL development in patients. Apart from these achievements, there are still important gaps in our knowledge of the epigenetic features of Tfh cells. Further studies will be required to draw a comprehensive epigenetic landscape of Tfh cells and identify potential candidate chromatin modifiers that participate in Tfh development. Understanding these issues and dissecting the underlying regulatory mechanisms will advance our knowledge of Tfh cells and shed lights on designing new strategies against those diseases associated with Tfh abnormalities.

ETHICS STATEMENT

All of our studies were specifically reviewed and approved by the Institutional Animal Care and Use Committees of the Third Military Medical University.

AUTHOR CONTRIBUTIONS

QH, JH, and JT wrote and edited the manuscript with LX and LY.

ACKNOWLEDGMENTS

We thank all the lab members from LY's lab for discussing this manuscript. This study was supported by grants from the National Key Research Development Plan of China (No. 2016YFA0502202 to LY) and the National Natural Science Foundation of China (No. 31700774 to LX).

REFERENCES

- Hashimoto M, Kamphorst AO, Im SJ, Kissick HT, Pillai RN, Ramalingam SS, et al. CD8 T cell exhaustion in chronic infection and cancer: opportunities for interventions. *Annu Rev Med.* (2018) 69:301–18. doi: 10.1146/annurev-med-012017-43208
- Hale JS, Youngblood B, Latner DR, Mohammed AU, Ye L, Akondy RS, et al. Distinct memory CD4⁺ T cells with commitment to T follicular helper- and T helper 1-cell lineages are generated after acute viral infection. *Immunity* (2013) 38:805–17. doi: 10.1016/j.immuni.2013.02.020
- Breitfeld D, Ohl L, Kremmer E, Ellwart J, Sallusto F, Lipp M, et al. Follicular B helper T cells express CXC chemokine receptor 5, localize to B cell follicles, and support immunoglobulin production. *J Exp Med.* (2000) 192:1545–52. doi: 10.1084/jem.192.11.1545
- Schaerli P, Willmann K, Lang AB, Lipp M, Loetscher P, Moser B. CXC chemokine receptor 5 expression defines follicular homing T cells with B cell helper function. *J Exp Med.* (2000) 192:1553–62. doi: 10.1084/jem.192.11.1553
- Crotty S. T follicular helper cell differentiation, function, and roles in disease. *Immunity* (2014) 41:529–42. doi: 10.1016/j.immuni.2014.10.004
- Crotty S. Follicular helper CD4 T cells (TFH). *Ann Rev Immunol.* (2011) 29:621–63. doi: 10.1146/annurev-immunol-031210-101400
- Johnston RJ, Poholek AC, DiToro D, Yusuf I, Eto D, Barnett B, et al. Bcl6 and Blimp-1 are reciprocal and antagonistic regulators of T follicular helper cell differentiation. *Science* (2009) 325:1006–10. doi: 10.1126/science.1175870
- Nurieva RI, Chung Y, Martinez GJ, Yang XO, Tanaka S, Matskevitch TD, et al. Bcl6 mediates the development of T follicular helper cells. *Science* (2009) 325:1001–5. doi: 10.1126/science.1176676
- Yu D, Rao S, Tsai LM, Lee SK, He Y, Sutcliffe EL, et al. The transcriptional repressor Bcl-6 directs T follicular helper cell lineage commitment. *Immunity* (2009) 31:457–68. doi: 10.1016/j.immuni.2009.07.002
- Baumjohann D, Okada T, Ansel KM. Cutting edge: distinct waves of BCL6 expression during T follicular helper cell development. *J Immunol.* (2011) 187:2089–92. doi: 10.4049/jimmunol.1101393
- Choi YS, Kageyama R, Eto D, Escobar TC, Johnston RJ, Monticelli L, et al. ICOS receptor instructs T follicular helper cell versus effector cell differentiation via induction of the transcriptional repressor Bcl6. *Immunity* (2011) 34:932–46. doi: 10.1016/j.immuni.2011.03.023
- Haynes NM, Allen CD, Lesley R, Ansel KM, Killeen N, Cyster JG. Role of CXCR5 and CCR7 in follicular Th cell positioning and appearance of a programmed cell death gene-1-high germinal center-associated subpopulation. *J Immunol.* (2007) 179:5099–108. doi: 10.4049/jimmunol.179.8.5099
- Xu H, Li X, Liu D, Li J, Zhang X, Chen X, et al. Follicular T-helper cell recruitment governed by bystander B cells and ICOS-driven motility. *Nature* (2013) 496:523–7. doi: 10.1038/nature12058
- Choi YS, Yang JA, Crotty S. Dynamic regulation of Bcl6 in follicular helper CD4 T (Tfh) cells. *Curr Opin Immunol.* (2013) 25:366–72. doi: 10.1016/j.coi.2013.04.003
- Schultz BT, Teigler JE, Pissani F, Oster AF, Kranias G, Alter G, et al. Circulating HIV-specific interleukin-21(+)CD4(+) T cells represent peripheral Tfh cells with antigen-dependent helper functions. *Immunity* (2016) 44:167–78. doi: 10.1016/j.immuni.2015.12.011
- Chahroudi A, Silvestri G. HIV and Tfh cells: circulating new ideas to identify and protect. *Immunity* (2016) 44:16–8. doi: 10.1016/j.immuni.2015.12.016
- Locci M, Havenar-Daughton C, Landais E, Wu J, Kroenke MA, Arlehamm CL, et al. Human circulating PD-1+CXCR3-CXCR5+ memory Tfh cells are highly functional and correlate with broadly neutralizing HIV antibody responses. *Immunity* (2013) 39:758–69. doi: 10.1016/j.immuni.2013.08.031
- He J, Tsai LM, Leong YA, Hu X, Ma CS, Chevalier N, et al. Circulating precursor CCR7(lo)PD-1(hi) CXCR5(+) CD4(+) T cells indicate Tfh cell activity and promote antibody responses upon antigen reexposure. *Immunity* (2013) 39:770–81. doi: 10.1016/j.immuni.2013.09.007
- Morita R, Schmitt N, Bentebibel SE, Ranganathan R, Bourdery L, Zurawski G, et al. Human blood CXCR5(+)CD4(+) T cells are counterparts of T follicular cells and contain specific subsets that differentially support antibody secretion. *Immunity* (2011) 34:108–21. doi: 10.1016/j.immuni.2010.12.012
- Ray JP, Staron MM, Shyer JA, Ho PC, Marshall HD, Gray SM, et al. The interleukin-2-mTORC1 kinase axis defines the signaling, differentiation, and metabolism of T helper 1 and follicular B helper T cells. *Immunity* (2015) 43:690–702. doi: 10.1016/j.immuni.2015.08.017
- Vinuesa CG, Linterman MA, Yu D, MacLennan IC. Follicular helper T cells. *Ann Rev Immunol.* (2016) 34:335–68. doi: 10.1146/annurev-immunol-041015-055605
- Qi H. T follicular helper cells in space-time. *Nat Rev Immunol.* (2016) 16:612–25. doi: 10.1038/nri.2016.94
- Kato Y, Zaid A, Davey GM, Mueller SN, Nutt SL, Zotos D, et al. Targeting antigen to Clec9A primes follicular Th cell memory responses capable of robust recall. *J Immunol.* (2015) 195:1006–14. doi: 10.4049/jimmunol.1500767
- Lahoud MH, Ahmet F, Kitsoulis S, Wan SS, Vremec D, Lee CN, et al. Targeting antigen to mouse dendritic cells via Clec9A induces potent CD4 T cell responses biased toward a follicular helper phenotype. *J Immunol.* (2011) 187:842–50. doi: 10.4049/jimmunol.1101176
- Stoll S, Delon J, Brotz TM, Germain RN. Dynamic imaging of T cell-dendritic cell interactions in lymph nodes. *Science* (2002) 296:1873–6. doi: 10.126/science.1071065
- Mempel TR, Henrickson SE, Von Andrian UH. T-cell priming by dendritic cells in lymph nodes occurs in three distinct phases. *Nature* (2004) 427:154–9. doi: 10.1038/nature02238
- Celli S, Lemaitre F, Bousso P. Real-time manipulation of T cell-dendritic cell interactions *in vivo* reveals the importance of prolonged contacts for CD4+ T cell activation. *Immunity* (2007) 27:625–34. doi: 10.1016/j.immuni.2007.08.018
- Eto D, Lao C, DiToro D, Barnett B, Escobar TC, Kageyama R, et al. IL-21 and IL-6 are critical for different aspects of B cell immunity and redundantly induce optimal follicular helper CD4 T cell (Tfh) differentiation. *PLoS ONE* (2011) 6:e17739. doi: 10.1371/journal.pone.0017739
- Ray JP, Marshall HD, Laidlaw BJ, Staron MM, Kaech SM, Craft J. Transcription factor STAT3 and type I interferons are corepressive insulators for differentiation of follicular helper and T helper 1 cells. *Immunity* (2014) 40:367–77. doi: 10.1016/j.immuni.2014.02.005
- Johnston RJ, Choi YS, Diamond JA, Yang JA, Crotty S. STAT5 is a potent negative regulator of TFH cell differentiation. *J Exp Med.* (2012) 209:243–50. doi: 10.1084/jem.20111174
- Oestreich KJ, Mohn SE, Weinmann AS. Molecular mechanisms that control the expression and activity of Bcl-6 in TH1 cells to regulate flexibility with a TFH-like gene profile. *Nat Immunol.* (2012) 13:405–11. doi: 10.1038/ni.2242
- Xu L, Cao Y, Xie Z, Huang Q, Bai Q, Yang X, et al. The transcription factor TCF-1 initiates the differentiation of T(FH) cells during acute viral infection. *Nat Immunol.* (2015) 16:991–9. doi: 10.1038/ni.3229
- Wu T, Shin HM, Moseman EA, Ji Y, Huang B, Harly C, et al. TCF1 is required for the T follicular helper cell response to viral infection. *Cell Rep.* (2015) 12:2099–110. doi: 10.1016/j.celrep.2015.08.049
- Choi YS, Gullicksrud JA, Xing S, Zeng Z, Shan Q, Li F, et al. LEF-1 and TCF-1 orchestrate T(FH) differentiation by regulating differentiation circuits upstream of the transcriptional repressor Bcl6. *Nat Immunol.* (2015) 16:980–90. doi: 10.1038/ni.3226
- Lee JY, Skon CN, Lee YJ, Oh S, Taylor JJ, Malhotra D, et al. The transcription factor KLF2 restrains CD4(+) T follicular helper cell differentiation. *Immunity* (2015) 42:252–64. doi: 10.1016/j.immuni.2015.01.013
- Weber JP, Fuhrmann F, Feist RK, Lahmann A, Al Baz MS, Gentz LJ, et al. ICOS maintains the T follicular helper cell phenotype by down-regulating Kruppel-like factor 2. *J Exp Med.* (2015) 212:217–33. doi: 10.1084/jem.20141432
- Weinstein JS, Laidlaw BJ, Lu Y, Wang JK, Schulz VP, Li N, et al. STAT4 and T-bet control follicular helper T cell development in viral infections. *J Exp Med.* (2018) 215:337–55. doi: 10.1084/jem.20170457
- Fang D, Cui K, Mao K, Hu G, Li R, Zheng M, et al. Transient T-bet expression functionally specifies a distinct T follicular helper subset. *J Exp Med.* (2018) 215:2705–14. doi: 10.1084/jem.20180927
- Shaw LA, Belanger S, Omilusik KD, Cho S, Scott-Browne JP, Nance JP, et al. Id2 reinforces TH1 differentiation and inhibits E2A to repress TFH differentiation. *Nat Immunol.* (2016) 17:834–43. doi: 10.1038/ni.3461

40. Ogbe A, Miao T, Symonds ALJ, Omodho B, Singh R, Bhullar P, et al. Early growth response genes 2 and 3 regulate the expression of Bcl6 and differentiation of T follicular helper cells. *J Biol Chem.* (2015) 290:20455–65. doi: 10.1074/jbc.M114.634816
41. Liu X, Chen X, Zhong B, Wang A, Wang X, Chu F, et al. Transcription factor achaete-scute homologue 2 initiates follicular T-helper-cell development. *Nature* (2014) 507:513–8. doi: 10.1038/nature12910
42. Shi B, Geng J, Wang YH, Wei H, Walters B, Li W, et al. Foxp1 negatively regulates T follicular helper cell differentiation and germinal center responses by controlling cell migration and CTLA-4. *J Immunol.* (2018) 200:586–94. doi: 10.4049/jimmunol.1701000
43. Stone EL, Pepper N, Katayama CD, Kerdiles YM, Lai CY, Emslie E, et al. ICOS coreceptor signaling inactivates the transcription factor FOXO1 to promote Tfh cell differentiation. *Immunity* (2015) 42:239–51. doi: 10.1016/j.immuni.2015.01.017
44. Auderset F, Schuster S, Fasnacht N, Coutaz M, Charmoy M, Koch U, et al. Notch signaling regulates follicular helper T cell differentiation. *J Immunol.* (2013) 191:2344–50. doi: 10.4049/jimmunol.1300643
45. Bollig N, Brustle A, Kellner K, Ackermann W, Abass E, Raifer H, et al. Transcription factor IRF4 determines germinal center formation through follicular T-helper cell differentiation. *Proc Natl Acad Sci USA.* (2012) 109:8664–9. doi: 10.1073/pnas.1205834109
46. Bauquet AT, Jin H, Paterson AM, Mitsdoerffer M, Ho IC, Sharpe AH, et al. The costimulatory molecule ICOS regulates the expression of c-Maf and IL-21 in the development of follicular T helper cells and TH-17 cells. *Nat Immunol.* (2009) 10:167–75. doi: 10.1038/ni.1690
47. Krishnamoorthy V, Kannanganat S, Maienschein-Cline M, Cook SL, Chen J, Bahros N, et al. The IRF4 gene regulatory module functions as a read-write integrator to dynamically coordinate T helper cell fate. *Immunity* (2017) 47:481–97.e7. doi: 10.1016/j.immuni.2017.09.001
48. Yang J, Lin X, Pan Y, Wang J, Chen P, Huang H, et al. Critical roles of mTOR Complex 1 and 2 for T follicular helper cell differentiation and germinal center responses. *eLife* (2016) 5:e17936. doi: 10.7554/eLife.17936
49. Zeng H, Cohen S, Guy C, Shrestha S, Neale G, Brown SA, et al. mTORC1 and mTORC2 kinase signaling and glucose metabolism drive follicular helper T cell differentiation. *Immunity* (2016) 45:540–54. doi: 10.1016/j.immuni.2016.08.017
50. Hao Y, Wang Y, Liu X, Yang X, Wang P, Tian Q, et al. The kinase complex mTOR complex 2 promotes the follicular migration and functional maturation of differentiated follicular helper CD4(+) T cells during viral infection. *Front Immunol.* (2018) 9:1127. doi: 10.3389/fimmu.2018.01127
51. Gunn MD, Ngo VN, Ansel KM, Ekland EH, Cyster JG, Williams LT. A B-cell-homing chemokine made in lymphoid follicles activates Burkitt's lymphoma receptor-1. *Nature* (1998) 391:799–803. doi: 10.1038/35876
52. Allen CD, Cyster JG. Follicular dendritic cell networks of primary follicles and germinal centers: phenotype and function. *Semin Immunol.* (2008) 20:14–25. doi: 10.1016/j.smim.2007.12.001
53. Rasheed AU, Rahn HP, Sallusto F, Lipp M, Muller G. Follicular B helper T cell activity is confined to CXCR5(hi)ICOS(hi) CD4 T cells and is independent of CD57 expression. *Eur J Immunol.* (2006) 36:1892–903. doi: 10.1002/eji.200636136
54. Kim CH, Lim HW, Kim JR, Rott L, Hillsamer P, Butcher EC. Unique gene expression program of human germinal center T helper cells. *Blood* (2004) 104:1952–60. doi: 10.1182/blood-2004-03-1206
55. Amanna IJ, Carlson NE, Slika MK. Duration of humoral immunity to common viral and vaccine antigens. *N Eng J Med.* (2007) 357:1903–15. doi: 10.1056/NEJMoa066092
56. Fazilleau N, Mark L, McHeyzer-Williams LJ, McHeyzer-Williams MG. Follicular helper T cells: lineage and location. *Immunity* (2009) 30:324–35. doi: 10.1016/j.immuni.2009.03.003
57. Crotty S, Kersh EN, Cannons J, Schwartzberg PL, Ahmed R. SAP is required for generating long-term humoral immunity. *Nature* (2003) 421:282–7. doi: 10.1038/nature01318
58. McCausland MM, Yusuf I, Tran H, Ono N, Yanagi Y, Crotty S. SAP regulation of follicular helper CD4 T cell development and humoral immunity is independent of SLAM and Fyn kinase. *J Immunol.* (2007) 178:817–28. doi: 10.4049/jimmunol.178.2.817
59. Kamperschroer C, Dibble JP, Meents DL, Schwartzberg PL, Swain SL. SAP is required for Th cell function and for immunity to influenza. *J Immunol.* (2006) 177:5317–27. doi: 10.4049/jimmunol.177.8.5317
60. Liu T, Chambers TJ. Yellow fever virus encephalitis: properties of the brain-associated T-cell response during virus clearance in normal and gamma interferon-deficient mice and requirement for CD4+ lymphocytes. *J Virol.* (2001) 75:2107–18. doi: 10.1128/jvi.75.5.2107-2118.2001
61. Sette A, Moutaftsi M, Moyron-Quiroz J, McCausland MM, Davies DH, Johnston RJ, et al. Selective CD4+ T cell help for antibody responses to a large viral pathogen: deterministic linkage of specificities. *Immunity* (2008) 28:847–58. doi: 10.1016/j.immuni.2008.04.018
62. Chen J, Lau YF, Lamirande EW, Paddock CD, Bartlett JH, Zaki SR, et al. Cellular immune responses to severe acute respiratory syndrome coronavirus (SARS-CoV) infection in senescent BALB/c mice: CD4+ T cells are important in control of SARS-CoV infection. *J Virol.* (2010) 84:1289–301. doi: 10.1128/jvi.01281-09
63. Thomsen AR, Nansen A, Andersen C, Johansen J, Marker O, Christensen JP. Cooperation of B cells and T cells is required for survival of mice infected with vesicular stomatitis virus. *Int Immunol.* (1997) 9:1757–66.
64. Borrow P, Tishon A, Lee S, Xu J, Grewal IS, Oldstone MB, et al. CD40L-deficient mice show deficits in antiviral immunity and have an impaired memory CD8+ CTL response. *J Exp Med.* (1996) 183:2129–42.
65. Edelmann KH, Wilson CB. Role of CD28/CD80-86 and CD40/CD154 costimulatory interactions in host defense to primary herpes simplex virus infection. *J Virol.* (2001) 75:612–21. doi: 10.1128/jvi.75.2.612-621.2001
66. Sangster MY, Riberdy JM, Gonzalez M, Topham DJ, Baumgarth N, Doherty PC. An early CD4+ T cell-dependent immunoglobulin A response to influenza infection in the absence of key cognate T-B interactions. *J Exp Med.* (2003) 198:1011–21. doi: 10.1084/jem.20021745
67. Bertram EM, Tafuri A, Shahinian A, Chan VS, Hunziker L, Recher M, et al. Role of ICOS versus CD28 in antiviral immunity. *Eur J Immunol.* (2002) 32:3376–85. doi: 10.1002/1521-4141(200212)32:12<3376::aid-immu3376>3.0.co;2-y
68. Hatzl K, Nance JP, Kroenke MA, Bothwell M, Haddad EK, Melnick A, et al. BCL6 orchestrates Tfh cell differentiation via multiple distinct mechanisms. *J Exp Med.* (2015) 212:539–53. doi: 10.1084/jem.20141380
69. Kroenke MA, Eto D, Locci M, Cho M, Davidson T, Haddad EK, et al. Bcl6 and Maf cooperate to instruct human follicular helper CD4 T cell differentiation. *J Immunol.* (2012) 188:3734–44. doi: 10.4049/jimmunol.1103246
70. Mueller SN, Gebhardt T, Carbone FR, Heath WR. Memory T cell subsets, migration patterns, and tissue residence. *Ann Rev Immunol.* (2013) 31:137–61. doi: 10.1146/annurev-immunol-032712-095954
71. McKinstry KK, Strutt TM, Swain SL. The effector to memory transition of CD4 T cells. *Immunol Res.* (2008) 40:114–27. doi: 10.1007/s12026-007-8004-y
72. Weber JP, Fuhrmann F, Hutloff A. T-follicular helper cells survive as long-term memory cells. *Eur J Immunol.* (2012) 42:1981–8. doi: 10.1002/eji.201242540
73. Choi YS, Yang JA, Yusuf I, Johnston RJ, Greenbaum J, Peters B, et al. Bcl6 expressing follicular helper CD4 T cells are fate committed early and have the capacity to form memory. *J Immunol.* (2013) 190:4014–26. doi: 10.4049/jimmunol.1202963
74. Liu X, Yan X, Zhong B, Nurieva RI, Wang A, Wang X, et al. Bcl6 expression specifies the T follicular helper cell program *in vivo*. *J Exp Med.* (2012) 209:1841–52. doi: 10.1084/jem.20120219
75. Bentebibel SE, Lopez S, Obermoser G, Schmitt N, Mueller C, Harrod C, et al. Induction of ICOS+CXCR3+CXCR5+ TH cells correlates with antibody responses to influenza vaccination. *Sci Transl Med.* (2013) 5:176ra32. doi: 10.1126/scitranslmed.3005191
76. MacLeod MK, David A, McKee AS, Crawford F, Kappler JW, Marrack P. Memory CD4 T cells that express CXCR5 provide accelerated help to B cells. *J Immunol.* (2011) 186:2889–96. doi: 10.4049/jimmunol.1002955
77. Shulman Z, Gitlin AD, Targ S, Jankovic M, Pasqual G, Nussenzweig MC, et al. T follicular helper cell dynamics in germinal centers. *Science* (2013) 341:673–7. doi: 10.1126/science.1241680

78. Kitano M, Moriyama S, Ando Y, Hikida M, Mori Y, Kurosaki T, et al. Bcl6 protein expression shapes pre-germinal center B cell dynamics and follicular helper T cell heterogeneity. *Immunity* (2011) 34:961–72. doi: 10.1016/j.immuni.2011.03.025
79. Iyer SS, Latner DR, Zilliox MJ, McCausland M, Akondy RS, Penaloza-Macmaster P, et al. Identification of novel markers for mouse CD4(+) T follicular helper cells. *Eur J Immunol.* (2013) 43:3219–32. doi: 10.1002/eji.201343469
80. Hale JS, Ahmed R. Memory T follicular helper CD4 T cells. *Front Immunol.* (2015) 6:16. doi: 10.3389/fimmu.2015.00016
81. Fazilleau N, McHeyzer-Williams LJ, Rosen H, McHeyzer-Williams MG. The function of follicular helper T cells is regulated by the strength of T cell antigen receptor binding. *Nat Immunol.* (2009) 10:375–84. doi: 10.1038/ni.1704
82. Tubo NJ, Pagan AJ, Taylor JJ, Nelson RW, Linehan JL, Ertelt JM, et al. Single naive CD4+ T cells from a diverse repertoire produce different effector cell types during infection. *Cell* (2013) 153:785–96. doi: 10.1016/j.cell.2013.04.007
83. Kim C, Wilson T, Fischer KE, Williams MA. Sustained interactions between T cell receptors and antigens promote the differentiation of CD4(+) memory T cells. *Immunity* (2013) 39:508–20. doi: 10.1016/j.immuni.2013.08.033
84. Koguchi Y, Thauland TJ, Slika MK, Parker DC. Preformed CD40 ligand exists in secretory lysosomes in effector and memory CD4+ T cells and is quickly expressed on the cell surface in an antigen-specific manner. *Blood* (2007) 110:2520–7. doi: 10.1182/blood-2007-03-081299
85. Fazilleau N, Eisenbraun MD, Malherbe L, Ebright JN, Pogue-Caley RR, McHeyzer-Williams LJ, et al. Lymphoid reservoirs of antigen-specific memory T helper cells. *Nat Immunol.* (2007) 8:753–61. doi: 10.1038/ni1472
86. Wilson JA, Hevey M, Bakken R, Guest S, Bray M, Schmaljohn AL, et al. Epitopes involved in antibody-mediated protection from Ebola virus. *Science* (2000) 287:1664–6. doi: 10.1126/science.287.5458.1664
87. Hofmeister Y, Planitzer CB, Farcet MR, Teschner W, Butterweck HA, Weber A, et al. Human IgG subclasses: in vitro neutralization of and in vivo protection against West Nile virus. *J Virol.* (2011) 85:1896–9. doi: 10.1128/jvi.02155-10
88. Huber VC, McKeon RM, Brackin MN, Miller LA, Keating R, Brown SA, et al. Distinct contributions of vaccine-induced immunoglobulin G1 (IgG1) and IgG2a antibodies to protective immunity against influenza. *Clin Vacc Immunol.* (2006) 13:981–90. doi: 10.1128/cvi.00156-06
89. Brooks DG, Teyton L, Oldstone MB, McGavern DB. Intrinsic functional dysregulation of CD4 T cells occurs rapidly following persistent viral infection. *J Virol.* (2005) 79:10514–27. doi: 10.1128/jvi.79.16.10514-10527.2005
90. Han S, Asoyan A, Rabenstein H, Nakano N, Obst R. Role of antigen persistence and dose for CD4+ T-cell exhaustion and recovery. *Proc Natl Acad Sci USA.* (2010) 107:20453–8. doi: 10.1073/pnas.1008437107
91. Fahey LM, Wilson EB, Elsaesser H, Fistonich CD, McGavern DB, Brooks DG. Viral persistence redirects CD4 T cell differentiation toward T follicular helper cells. *J Exp Med.* (2011) 208:987–99. doi: 10.1084/jem.20101773
92. Crawford A, Angelosanto JM, Kao C, Doering TA, Odorizzi PM, Barnett BE, et al. Molecular and transcriptional basis of CD4(+) T cell dysfunction during chronic infection. *Immunity* (2014) 40:289–302. doi: 10.1016/j.immuni.2014.01.005
93. Greczmiel U, Krautler NJ, Pedrioli A, Bartsch I, Agnellini P, Bedenikovic G, et al. Sustained T follicular helper cell response is essential for control of chronic viral infection. *Sci Immunol.* (2017) 2:aam8686. doi: 10.1126/sciimmunol.aam8686
94. Wilson EB, Yamada DH, Elsaesser H, Herskovitz J, Deng J, Cheng G, et al. Blockade of chronic type I interferon signaling to control persistent LCMV infection. *Science* (2013) 340:202–7. doi: 10.1126/science.1235208
95. Osokine I, Snell LM, Cunningham CR, Yamada DH, Wilson EB, Elsaesser HJ, et al. Type I interferon suppresses *de novo* virus-specific CD4 Th1 immunity during an established persistent viral infection. *Proc Natl Acad Sci USA.* (2014) 111:7409–14. doi: 10.1073/pnas.1401662111
96. Snell LM, Brooks DG. New insights into type I interferon and the immunopathogenesis of persistent viral infections. *Curr Opin Immunol.* (2015) 34:91–8. doi: 10.1016/j.coi.2015.03.002
97. Teijaro JR, Ng C, Lee AM, Sullivan BM, Sheehan KC, Welch M, et al. Persistent LCMV infection is controlled by blockade of type I interferon signaling. *Science* (2013) 340:207–11. doi: 10.1126/science.1235214
98. Harker JA, Dolgote A, Zuniga EI. Cell-intrinsic IL-27 and gp130 cytokine receptor signaling regulates virus-specific CD4(+) T cell responses and viral control during chronic infection. *Immunity* (2013) 39:548–59. doi: 10.1016/j.immuni.2013.08.010
99. Harker JA, Lewis GM, Mack L, Zuniga EI. Late interleukin-6 escalates T follicular helper cell responses and controls a chronic viral infection. *Science* (2011) 334:825–9. doi: 10.1126/science.1208421
100. Raju S, Kometani K, Kurosaki T, Shaw AS, Egawa T. The adaptor molecule CD2AP in CD4 T cells modulates differentiation of follicular helper T cells during chronic LCMV infection. *PLoS Pathog.* (2018) 14:e1007053. doi: 10.1371/journal.ppat.1007053
101. Chung Y, Tanaka S, Chu F, Nurieva RI, Martinez GJ, Rawal S, et al. Follicular regulatory T cells expressing Foxp3 and Bcl-6 suppress germinal center reactions. *Nat Med.* (2011) 17:983–8. doi: 10.1038/nm.2426
102. Linterman MA, Pierson W, Lee SK, Kallies A, Kawamoto S, Rayner TF, et al. Foxp3+ follicular regulatory T cells control the germinal center response. *Nat Med.* (2011) 17:975–82. doi: 10.1038/nm.2425
103. Penaloza-MacMaster P, Kamphorst AO, Wieland A, Araki K, Iyer SS, West EE, et al. Interplay between regulatory T cells and PD-1 in modulating T cell exhaustion and viral control during chronic LCMV infection. *J Exp Med.* (2014) 211:1905–18. doi: 10.1084/jem.20132577
104. Leong YA, Chen Y, Ong HS, Wu D, Man K, Deleage C, et al. CXCR5+ follicular cytotoxic T cells control viral infection in B cell follicles. *Nat Immunol.* (2016) 17:1187–96. doi: 10.1038/ni.3543
105. Im SJ, Hashimoto M, Gerner MY, Lee J, Kissick HT, Burger MC, et al. Defining CD8+ T cells that provide the proliferative burst after PD-1 therapy. *Nature* (2016) 537:417–21. doi: 10.1038/nature19330
106. He R, Hou S, Liu C, Zhang A, Bai Q, Han M, et al. Follicular CXCR5-expressing CD8+ T cells curtail chronic viral infection. *Nature* (2016) 537:412–28. doi: 10.1038/nature19317
107. Elsaesser H, Sauer K, Brooks DG. IL-21 is required to control chronic viral infection. *Science* (2009) 324:1569–72. doi: 10.1126/science.1174182
108. Frohlich A, Kisielow J, Schmitz I, Freigang S, Shamshiev AT, Weber J, et al. IL-21R on T cells is critical for sustained functionality and control of chronic viral infection. *Science* (2009) 324:1576–80. doi: 10.1126/science.1172815
109. Yi JS, Du M, Zajac AJ. A vital role for interleukin-21 in the control of a chronic viral infection. *Science* (2009) 324:1572–6. doi: 10.1126/science.1175194
110. Chevalier ME, Julg B, Pyo A, Flanders M, Ranasinghe S, Soghoian DZ, et al. HIV-1-specific interleukin-21+ CD4+ T cell responses contribute to durable viral control through the modulation of HIV-specific CD8+ T cell function. *J Virol.* (2011) 85:733–41. doi: 10.1128/jvi.02030-10
111. Battegay M, Moskopidis D, Waldner H, Brundler MA, Fung-Leung WP, Mak TW, et al. Impairment and delay of neutralizing antiviral antibody responses by virus-specific cytotoxic T cells. *J Immunol.* (1993) 151:5408–15.
112. Hunziker L, Recher M, Macpherson AJ, Ciurea A, Freigang S, Hengartner H, et al. Hypergammaglobulinemia and autoantibody induction mechanisms in viral infections. *Nat Immunol.* (2003) 4:343–9. doi: 10.1038/ni911
113. Buchmeier MJ, Oldstone MB. Virus-induced immune complex disease: identification of specific viral antigens and antibodies deposited in complexes during chronic lymphocytic choriomeningitis virus infection. *J Immunol.* (1978) 120:1297–304.
114. Lindqvist M, van Lunzen J, Soghoian DZ, Kuhl BD, Ranasinghe S, Kranias G, et al. Expansion of HIV-specific T follicular helper cells in chronic HIV infection. *J Clin Invest.* (2012) 122:3271–80. doi: 10.1172/jci64314
115. Cubas RA, Mudd JC, Savoye AL, Perreau M, van Grevenynghe J, Metcalf T, et al. Inadequate T follicular cell help impairs B cell immunity during HIV infection. *Nat Med.* (2013) 19:494–9. doi: 10.1038/nm.3109
116. Petrovas C, Yamamoto T, Gerner MY, Boswell KL, Wloka K, Smith EC, et al. CD4 T follicular helper cell dynamics during SIV infection. *J Clin Invest.* (2012) 122:3281–94. doi: 10.1172/jci63039
117. Xiao M, Chen X, He R, Ye L. Differentiation and function of follicular CD8 T cells during human immunodeficiency virus infection. *Front Immunol.* (2018) 9:1095. doi: 10.3389/fimmu.2018.01095

118. Kohler SL, Pham MN, Folkvord JM, Arends T, Miller SM, Miles B, et al. Germinal center T follicular helper cells are highly permissive to HIV-1 and alter their phenotype during virus replication. *J Immunol.* (2016) 196:2711–22. doi: 10.4049/jimmunol.1502174
119. Banga R, Procopio FA, Noto A, Pollakis G, Cavassini M, Ohmiti K, et al. PD-1(+) and follicular helper T cells are responsible for persistent HIV-1 transcription in treated aviremic individuals. *Nat Med.* (2016) 22:754–61. doi: 10.1038/nm.4113
120. Perreau M, Savoye AL, De Crignis E, Corpataux JM, Cubas R, Haddad EK, et al. Follicular helper T cells serve as the major CD4 T cell compartment for HIV-1 infection, replication, and production. *J Exp Med.* (2013) 210:143–56. doi: 10.1084/jem.20121932
121. Hufert FT, van Lunzen J, Janossy G, Bertram S, Schmitz J, Haller O, et al. Germinal centre CD4⁺ T cells are an important site of HIV replication *in vivo*. *AIDS* (1997) 11:849–57.
122. Streeck H, D'Souza MP, Littman DR, Crotty S. Harnessing CD4(+) T cell responses in HIV vaccine development. *Nat Med.* (2013) 19:143–9. doi: 10.1038/nm.3054
123. Lane HC, Masur H, Edgar LC, Whalen G, Rook AH, Fauci AS. Abnormalities of B-cell activation and immunoregulation in patients with the acquired immunodeficiency syndrome. *N Engl J Med.* (1983) 309:453–8. doi: 10.1056/nejm198308253090803
124. Wendel BS, Del Alcazar D, He C, Del Rio-Estrada PM, Aiamkitsumrit B, Ablanedo-Terrazas Y, et al. The receptor repertoire and functional profile of follicular T cells in HIV-infected lymph nodes. *Sci Immunol.* (2018) 3:eaan8884. doi: 10.1126/sciimmunol.aan8884
125. Vella LA, Herati RS, Wherry EJ. CD4(+) T cell differentiation in chronic viral infections: the Tfh perspective. *Trends Mol Med.* (2017) 23:1072–87. doi: 10.1016/j.molmed.2017.10.001
126. Bergthaler A, Flatz L, Verschoor A, Hegazy AN, Holdener M, Fink K, et al. Impaired antibody response causes persistence of prototypic T cell-contained virus. *PLoS Biol.* (2009) 7:e1000080. doi: 10.1371/journal.pbio.1000080
127. Odorizzi PM, Wherry EJ. Inhibitory receptors on lymphocytes: insights from infections. *J Immunol.* (2012) 188:2957–65. doi: 10.4049/jimmunol.1100038
128. Wykes MN, Lewin SR. Immune checkpoint blockade in infectious diseases. *Nat Rev Immunol.* (2017) 18:91–104. doi: 10.1038/nri.2017.112
129. McNeil AC, Shupert WL, Iyasere CA, Hallahan CW, Mican JA, Davey RT Jr, et al. High-level HIV-1 viremia suppresses viral antigen-specific CD4(+) T cell proliferation. *Proc Natl Acad Sci USA.* (2001) 98:13878–83. doi: 10.1073/pnas.251539598
130. Hryniewicz A, Boasso A, Edgill-Smith Y, Vaccari M, Fuchs D, Venzon D, et al. CTLA-4 blockade decreases TGF-beta, IDO, and viral RNA expression in tissues of SIVmac251-infected macaques. *Blood* (2006) 108:3834–42. doi: 10.1182/blood-2006-04-010637
131. Jones RB, Ndhlovu LC, Barbour JD, Sheth PM, Jha AR, Long BR, et al. Tim-3 expression defines a novel population of dysfunctional T cells with highly elevated frequencies in progressive HIV-1 infection. *J Exp Med.* (2008) 205:2763–79. doi: 10.1084/jem.20081398
132. Day CL, Kaufmann DE, Kiepiela P, Brown JA, Moodley ES, Reddy S, et al. PD-1 expression on HIV-specific T cells is associated with T-cell exhaustion and disease progression. *Nature* (2006) 443:350–4. doi: 10.1038/nature05115
133. Aubert RD, Kamphorst AO, Sarkar S, Vezys V, Ha SJ, Barber DL, et al. Antigen-specific CD4 T-cell help rescues exhausted CD8 T cells during chronic viral infection. *Proc Natl Acad Sci USA.* (2011) 108:21182–7. doi: 10.1073/pnas.1118450109
134. He R, Yang X, Liu C, Chen X, Wang L, Xiao M, et al. Efficient control of chronic LCMV infection by a CD4 T cell epitope-based heterologous prime-boost vaccination in a murine model. *Cell Mol Immunol.* (2017) 15:815–26. doi: 10.1038/cmi.2017.3
135. Frebel H, Richter K, Oxenius A. How chronic viral infections impact on antigen-specific T-cell responses. *Eur J Immunol.* (2010) 40:654–63. doi: 10.1002/eji.200940102
136. Klenerman P, Hill A. T cells and viral persistence: lessons from diverse infections. *Nat Immunol.* (2005) 6:873–9. doi: 10.1038/ni1241
137. Good-Jacobson KL, Szumilas CG, Chen L, Sharpe AH, Tomayko MM, Shlomchik MJ. PD-1 regulates germinal center B cell survival and the formation and affinity of long-lived plasma cells. *Nat Immunol.* (2010) 11:535–42. doi: 10.1038/ni.877
138. Barton E, Mandal P, Speck SH. Pathogenesis and host control of gammaherpesviruses: lessons from the mouse. *Annu Rev Immunol.* (2011) 29:351–97. doi: 10.1146/annurev-immunol-072710-81639
139. Flano E, Husain SM, Sample JT, Woodland DL, Blackman MA. Latent murine gamma-herpesvirus infection is established in activated B cells, dendritic cells, and macrophages. *J Immunol.* (2000) 165:1074–81. doi: 10.4049/jimmunol.165.2.1074
140. Stevenson PG, Doherty PC. Non-antigen-specific B-cell activation following murine gammaherpesvirus infection is CD4 independent *in vitro* but CD4 dependent *in vivo*. *J Virol.* (1999) 73:1075–9.
141. Decalf J, Godinho-Silva C, Fontinha D, Marques S, Simas JP. Establishment of murine gammaherpesvirus latency in B cells is not a stochastic event. *PLoS Pathog.* (2014) 10:e1004269. doi: 10.1371/journal.ppat.1004269
142. Barton ES, Rajkarnikar S, Langston PK, Price MJ, Grayson JM. Gammaherpesvirus latency differentially impacts the generation of primary versus secondary memory CD8+ T cells during subsequent infection. *J Virol.* (2014) 88:12740–51. doi: 10.1128/JVI.02106-14
143. Collins CM, Speck SH. Expansion of murine gammaherpesvirus latently infected B cells requires T follicular help. *PLoS Pathog.* (2014) 10:e1004106. doi: 10.1371/journal.ppat.1004106
144. Sparks-Thissen RL, Braaten DC, Kreher S, Speck SH, Virgin HW. An optimized CD4 T-cell response can control productive and latent gammaherpesvirus infection. *J Virol.* (2004) 78:6827–35. doi: 10.1128/JVI.78.13.6827-35.2004
145. Mylvaganam GH, Rios D, Abdelaal HM, Iyer S, Tharp G, Mavigner M, et al. Dynamics of SIV-specific CXCR5+ CD8 T cells during chronic SIV infection. *Proc Natl Acad Sci USA.* (2017) 114:1976–81. doi: 10.073/pnas.1621418114
146. Wilson CB, Rowell E, Sekimata M. Epigenetic control of T-helper-cell differentiation. *Nat Rev Immunol.* (2009) 9:91–105. doi: 10.1038/nri2487
147. Wei G, Wei L, Zhu J, Zang C, Hu-Li J, Yao Z, et al. Global mapping of H3K4me3 and H3K27me3 reveals specificity and plasticity in lineage fate determination of differentiating CD4+ T cells. *Immunity* (2009) 30:155–67. doi: 10.1016/j.immuni.2008.12.009
148. Cook KD, Shpargel Karl B, Starmer J, Whitfield-Larry F, Conley B, Allard Denise E, et al. T follicular helper cell-dependent clearance of a persistent virus infection requires T cell expression of the histone demethylase UTX. *Immunity* (2015) 43:703–14. doi: 10.1016/j.immuni.2015.09.002
149. Nishizawa S, Sakata-Yanagimoto M, Hattori K, Muto H, Nguyen T, Izutsu K, et al. BCL6 locus is hypermethylated in angioimmunoblastic T-cell lymphoma. *Int J Hematol.* (2016) 105:465–9. doi: 10.1007/s12185-016-2159-z

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2019 Huang, Hu, Tang, Xu and Ye. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



Interleukin-1 in the Response of Follicular Helper and Follicular Regulatory T Cells

Paul-Gydeon Ritvo¹ and David Klatzmann^{1,2*}

¹ Sorbonne Université, INSERM, Immunology-Immunopathology-Immunotherapy (i3), Paris, France, ² AP-HP, Hôpital Pitié-Salpêtrière, Biotherapy (CIC-BTi) and Inflammation-Immunopathology-Biotherapy Department (i2B), Paris, France

The role of interleukin-1 in the regulation of humoral responses is poorly documented, in contrast to its role in inflammation. Recent findings suggest there is an interleukin-1 axis in the follicular T cell control of B cell responses, involving interleukin-1 receptors (IL-1R1 and IL-1R2) and receptor antagonists (IL-1Ra). Here, we revisit the literature on this topic and conclude that targeting the interleukin-1 pathway should be a valuable therapeutic approach in many diseases involving excessive production of (auto)antibodies, such as autoimmune diseases or allergy.

Keywords: plasma cell, antibody production, immunoregulation, immunotherapy, germinal centers, Tfr cells, Tfh cells

OPEN ACCESS

Edited by:

Maria Pia Cicalese,
San Raffaele Scientific Institute
(IRCCS), Italy

Reviewed by:

Massimo Gadina,
National Institute of Arthritis and
Musculoskeletal and Skin Diseases
(NIAMS), United States
Michael Loran Dustin,
University of Oxford, United Kingdom

*Correspondence:

David Klatzmann
david.klatzmann@
sorbonne-universite.fr

Specialty section:

This article was submitted to
T Cell Biology,
a section of the journal
Frontiers in Immunology

Received: 27 July 2018

Accepted: 29 January 2019

Published: 27 February 2019

Citation:

Ritvo P-G and Klatzmann D (2019)
Interleukin-1 in the Response of
Follicular Helper and Follicular
Regulatory T Cells.
Front. Immunol. 10:250.
doi: 10.3389/fimmu.2019.00250

INTRODUCTION

Interleukin-1 (IL-1) is known as the key cytokine of innate immune responses and has been described as the “quintessential inflammatory cytokine” (1). IL-1 is predominantly produced by monocytes and macrophages (2, 3) following an external stimulus such as through Toll-Like Receptor (TLR) activation. IL-1 pleiotropic functions have so far mainly been linked to inflammation, orchestrating a first line of defense against pathogens (4). IL-1 has systemic effects that trigger fever, cortisol production, and liver stimulation (with production of C-reactive and complement proteins) and local effects on innate and adaptive immune cell stimulation. The effects of IL-1 on innate immunity have been extensively studied and reviewed (4, 5). Those on adaptive immunity have been ascribed to a general amplification of T-cell responses (6) and to modulation of T cell plasticity toward Th17 cell differentiation (7, 8). Except for the known role of IL-1 in adjuvant activity (9), the involvement of IL-1 in the regulation of humoral response is poorly documented. Indeed, a thorough check of the literature, including multiple PubMed queries such as various combinations of “interleukin-1,” “IL-1,” “IL-1Ra,” “IL-1R1,” “IL-1R2,” “Tfh,” “Tfr” “follicular cells,” “humoral immunity,” “antibody production,” “autoantibody production,” and “germinal centers” did not identify relevant publications. We review here recent findings that highlight a key role of IL-1 in the regulation of follicular helper and follicular regulatory T cells, thereby controlling B cell responses.

THE IL-1 ACTIVATION PATHWAY

IL-1 β is part of a wide family of cytokines (IL-1 α , IL-1 β , IL-18, IL-33, IL-36), receptor antagonists (IL-1Ra, IL-36Ra, IL-38) and the anti-inflammatory IL-37. The IL-1 activation pathway has been reviewed elsewhere (10). Briefly, IL-1 is produced as an inactive precursor, pro-IL-1 β , in response to pathogen-specific signals. This stimulation of innate immune cells induces the formation of the

inflammasome, a molecular scaffold composed of many molecules such as NLRP3 (11). This key system activates caspase 1 (also called ICE for Interleukin-1 Converting Enzyme), an enzyme able to cleave pro-IL-1 β (12). It is worthy of note that the mechanism of IL-1 β secretion is not the conventional endoplasmic reticulum and Golgi route (13), but is not well understood yet and may depend on many parameters, such as stimulus strength and IL-1 requirement (14).

The signaling pathway following the interaction of IL-1 with its agonist receptor IL-1R1 has been described to be the same as many other signaling pathways, such as those triggered by the interaction of pathogenic components with TLR or of IL-33 with its receptor (10, 15). The first step consists of the recruitment of MyD88 to the receptors (16), and the cascade that follows—called the “canonical pathway”—which leads to the final activation of NF- κ B. This ultimately activates the expression of pro-inflammatory genes such as cytokines, chemokines and adhesion molecules (17).

IL-1R2 and IL-1Ra regulate the IL-1 β / IL-1R1 interaction. IL-1R2 has an extracellular domain structurally similar to that of IL-1R1 but which lacks the intracellular domains allowing signaling. It thus acts as a decoy receptor, capturing the IL-1 β and thereby preventing IL-1R1 stimulation (18). IL-1R2 is expressed at high levels by macrophages, neutrophils and B-cells (19). IL-1Ra is a cytokine that inhibits IL-1 function by binding to IL-1R1 without producing any agonist effects, thereby preventing IL-1 binding (20, 21).

IL-1 AND THE REGULATION OF TFH AND TFR CELLS

Antibody production by plasma cells is tightly regulated by follicular helper T (Tfh) cells. Help by Tfh cells is essential for the differentiation of B cells into antibody-producing plasma cells (22, 23). In contrast, follicular regulatory T (Tfr) cells negatively control humoral immune responses (24, 25). These cells are thought to be derived from regulatory T (Treg) cells (26, 27). Their mechanisms of action are poorly known and they are thought to act by regulating the help provided by Tfh cells to B cells. However, recent findings have shown that few Tfr cells are located within the germinal centers (GCs) of LNs, where Tfh cells and plasma cells interact. Most Tfr cells are found surrounding the GCs and are likely not in contact with Tfh cells (28).

In contrast to Treg cells from which they are derived, we observed that Tfr cells do not respond to interleukin-2 (IL-2) (29). This led us to reexamine their phenotype thoroughly. In contrast to Treg cells and to previous description of the Tfr-cell phenotype, we showed that Tfr cells do not express IL-2Ra (CD25), the essential component of the high-affinity IL-2R. This is important because most previous investigations of Tfr cell biology actually reported the biology of mixtures of Tfr and Treg cells. The stringent characterization of Tfr cells allowed us to reveal a striking distribution of IL-1 receptor expression on Tfh and Tfr cells. We observed that Tfh cells express the IL-1R1 agonist receptor while Tfr cells express both the IL-1R2 decoy receptor and the antagonist IL-1Ra. The lack of CD25 expression

by Tfr cells and this distribution of IL-1 receptors have also been observed by others (30, 31).

This striking distribution of the agonist receptor on Tfh cells and of the antagonist receptors/inhibitors on Tfr cells led us to hypothesize and explore a possible IL-1 axis in the regulation of humoral responses. We observed that, *in vitro*, IL-1 β activated the production of IL-4 and IL-21 by Tfh cells. These cytokines have been shown to be crucial for the T-cell help to B cells (32). This cytokine production was suppressed by Tfr cells to the same extent as by recombinant IL-1Ra (Anakinra), indicating that the suppressive effect was likely dependent on the blocking of IL-1 by IL-1R2 on the surface of Tfr cells, or on IL-1Ra produced by Tfr cells. Eventually, we showed that, *in vivo*, IL-1 β induced proliferation of Tfh cells while Anakinra significantly reduced the proportion of Tfh cells.

Altogether, we revealed an IL-1 axis regulating the germinal center responses (29) and suggested the existence of a dual regulation of T cells in secondary lymphoid organs, one between Treg and effector T cells regulated by IL-2 outside GCs and the other between Tfh and Tfr cells regulated by IL-1 inside GCs.

IL-1 AND REGULATION OF THE HUMORAL RESPONSE (FIGURE 1)

There are no or few experiments that have investigated a direct link between IL-1 and antibody production. However, revisiting the literature, there are actually many observations indirectly supporting the involvement of an IL-1 axis in the control of humoral immunity (summarized in **Table 1**). First, IL-1 administration during an immunization enhanced humoral responses and led to greater antibody production (9, 38, 39), a phenomenon referred to as the “adjuvanticity of IL-1,” which was mostly thought to act by stimulation of innate immune cells that in turn would stimulate helper T cells to help B cells better. Interestingly, this observation is itself indirectly supported by the fact that many adjuvants used for immunization, such as the widely used alum, trigger enhanced IL-1 production (34). Similarly, stimulation by pathogens, which ultimately triggers antibody production, is also a strong IL-1 enhancer (35). Altogether, it appears that there is some positive correlation between production of IL-1 during immunization and the efficacy of the resulting B cell response.

Experiments using genetically modified mice not expressing the IL-1 gene or its receptors further support the importance of an IL-1 axis in the control of humoral responses. Following appropriate stimulation, IL-1-deficient mice produced significantly reduced amounts of antibodies compared to wild-type mice (36, 37). Conversely, mice deficient in the expression of IL-1Ra, an antagonist of the IL-1R1 receptor, showed increased antibody production in the same conditions (36, 37). In these studies, the effect of IL-1 on humoral immunity enhancement was shown to act through induction of co-stimulatory molecules on T cells, such as CD40L and OX40 (37), which were later found to be highly expressed on the surface of Tfh cells (40) (**Table 1**).

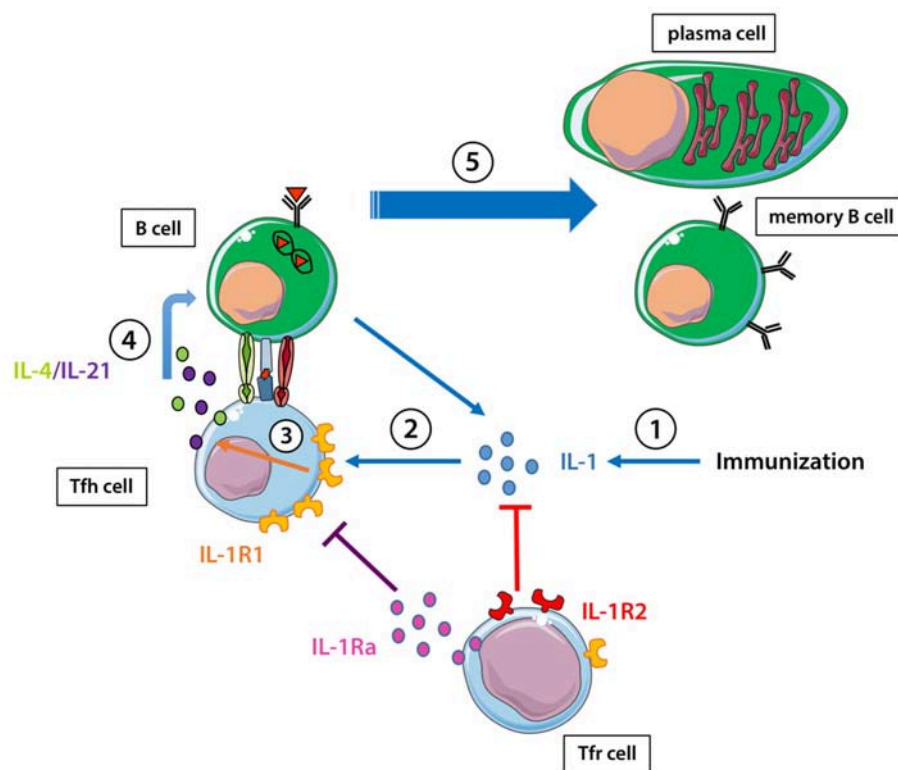


FIGURE 1 | IL-1 and the regulation of the humoral response. (1) Immunization enhances IL-1 production. (2) IL-1 stimulates tfh cells through the IL-1R1, can be captured by IL-1R2 on Tfr cells, and IL-1Ra produced by Tfr cells can block the action of IL-1 on IL-1R1. (3) IL-1 stimulates IL-4/IL-21 production by Tfh cells through its binding to IL-1R1. (4, 5) IL-4/IL-21 helps B cells to become either memory B cells or antibody-producing plasma cells.

IL-1 AND THE PATHOPHYSIOLOGY OF HUMORAL RESPONSES

Some evidence for the involvement of IL-1 in the humoral response can also be found by looking at diseases associated with excessive levels of antibodies and/or pathogenic antibodies.

Autoimmune Diseases

Autoantibody production is common in autoimmune diseases and frequently contributes to their pathophysiology. Much evidence of the involvement of IL-1 in the control of autoantibody production can be found in the recent literature.

One of the interesting models for the study of this role is Myasthenia Gravis (MG), a disease caused by a pathogenic anti-acetylcholine receptor (AChR) IgG1 (41). (i) IL-1 β gene polymorphisms have been found in association with MG, suggesting a possible pathogenic role of this cytokine in the disease (42); (ii) Anakinra reduced the clinical symptoms of mice with experimental autoimmune MG (EAMG) and suppressed the pathogenic anti-AChR IgG1 (41); (iii) inhibition of proteins involved in the production of IL-1 β , such as caspase-1, can regulate the humoral response in EAMG (43). In the classification by McGonagle and McDermott (44), MG is more an autoimmune than an auto-inflammatory disease. This suggests that the

consequences of blocking IL-1 in this disease should mostly be due not to the blocking of IL-1 inflammatory effects, but to the contribution of IL-1 to the regulation of antibody production.

Thyroid gland autoimmune disorders also appear informative. (i) A recent large-scale study found an association with the IL-1 RN (gene encoding the IL-1Ra) receptor antagonist variable number of tandem repeats (VNTR) polymorphism in Hashimoto Thyroiditis (HT) patients (45); (ii) increased percentages of circulating Tfh cells have been found in patients with autoimmune thyroid disorders, with a positive correlation between the percentages of circulating Tfh cells and the serum concentrations of anti-TSH receptor-Ab/thyroperoxidase-Ab/thyroglobulin-Ab (46); (iii) a study suggested that both promoter and exon polymorphisms of IL-1 β gene have a significant role in the risk of developing Graves' disease (GD) (47); (iv) although no significant differences in IL-1 β levels were found between serum from patients with HT or GD and normal controls, IL-1 β mRNA and protein levels in peripheral blood mononuclear cells of HT patients were found to be significantly higher than those of patients with GD, which were in turn higher than the level in normal controls; (v) IL-1 β mRNA was also increased in thyroid gland tissue from patients with HT compared to those with GD, and this was accompanied by increased local infiltration of monocytes into thyroid tissues; correlation analysis validated the association of

TABLE 1 | Experiments supporting a correlation between IL-1 production during immunization and resulting antibody production.

Experiment	Effect	Comment	References
IL-1 as an adjuvant	Enhanced antibody response	Dose- and time-dependent adjuvanticity of interleukin 1 <i>in vivo</i> . IL-1 given 2 h after the priming dose of antigen enhanced antibody response	(9)
IL-1 as an adjuvant	Enhanced antibody response	IL-1 is an effective mucosal vaccine adjuvant when coadministered with protein immunogens, and is as effective as cholera toxin in inducing Ag-specific serum IgG	(33)
Alum induces release of IL-1	Enhanced antibody response (alum effect)	Considering the immunostimulatory activities of these cytokines and the ability of IL-1 β to act as an adjuvant, the results suggest a mechanism for the adjuvanticity of alum	(34)
Cholera toxin induces release of IL-1	Enhanced antibody response	Cholera toxin (CT) is a strong systemic and mucosal adjuvant that greatly enhances IgG and IgA immune responses. It stimulates IL-1 production	(35)
IL-1 α/β -deficient mice	Reduced antibody production	Primary and secondary antibody production against T-dependent antigen was significantly reduced in IL-1 α/β ^{-/-} mice after immunization	(36, 37)
IL-1Ra-deficient mice	Increased antibody production	Primary and secondary antibody production against T-dependent antigen was significantly increased in IL-1Ra ^{-/-} mice after immunization	
IL-1 effects on CD40L	Increased CD40L expression	CD40L expression on T cells was affected in IL-1 ^{-/-} mice, and the reduced Ag-specific B cell response in IL-1 ^{-/-} mice was recovered by treatment with agonistic anti-CD40 mAb both <i>in vitro</i> and <i>in vivo</i> . IL-1 enhances T cell-dependent Ab production by augmenting CD40L	(37)

high IL-1 β levels with the pathogenesis of HT and led to the suggestion that IL-1 β may be an active etiologic factor in the pathogenesis of HT and thus represent a new target for novel diagnostics and treatment (48).

Rheumatoid or systemic diseases could also be studied from this point of view. For instance, in rheumatoid arthritis, anti-CCP antibodies were more frequently found in the rheumatoid arthritis subgroup with high levels of cytokines, including IL-1 (49). In systemic lupus erythematosus (SLE) models, mice deficient in the IL-1 β gene were found to be resistant to induction of experimental SLE and developed lower levels of anti-dsDNA antibodies, as compared to control mice (50). Bay11-7082—a broad-spectrum inhibitor with anti-inflammatory activity against multiple targets (51)—reduced autoantibody production and renal immune complex deposition in MRL/lpr mice via inhibiting NLRP3 inflammasome and NF- κ B activation (52). Compared to wild-type mice, caspase-1^{-/-} mice had significant reductions in both anti-dsDNA and anti-RNP autoantibody titers, abrogation of a type I IFN signature and were protected from both renal immune complex deposition and kidney inflammation (53). A few studies reported efficacy of IL-1 blockers in SLE patients (54–56), with documented decrease in anti-dsDNA antibody levels (54, 55).

In multiple sclerosis (MS), some antibodies may be involved in the pathophysiology of some form of the disease via demyelination (57). Among these antibodies, autoantibodies directed against lipids present in myelin (58), myelin oligodendrocyte glycoprotein (MOG) (59) or myelin basic protein (60) could be pathogenic, possibly through antibody deposition and complement activation, which are frequently found in chronic active lesions (61). Supporting this, new brain

lesions were reduced in MS patients receiving rituximab, an anti-CD20 drug that depletes B cells (62). Therapeutic plasma exchange has also been used to treat the disease, with success in the MS pattern involving prominent immunoglobulin and complement (63). On the other hand, IL-1 β expression in the central nervous system and in blood has been shown to be associated with disease activity, though direct mechanisms have not been established (64). IL-1R1-deficient mice were resistant to experimental autoimmune encephalomyelitis (EAE) (65), the mouse model of MS. Furthermore, treatment with IL-1Ra has some protective effect on rat EAE as it reduces the duration and severity of the disease (66). Altogether, this could be suggestive of an effect of the IL-1 axis on the disease through limitation of pathogenic antibody production in MS.

In celiac disease, individuals develop an immune reaction to gluten, mainly composed of IgA antibodies. Polymorphism of IL-1 has been associated with susceptibility to celiac disease (67) and IL-1 β is associated with the disease, though its mechanism of action is unknown (68). Finally, we recently showed that the TCR repertoires of Tfh and Tfr cells from spleens of immunised mice were surprisingly diverse and mostly composed of mildly expanded clonotypes suggesting a major bystander activation during the immune response in the GCs (69). It remains to investigate the contribution of IL-1 to this bystander activation and its possible relation to autoantibody formation.

Hypersensitivity and Allergy

Allergy also appears of interest in supporting the involvement of IL-1 in antibody production. In 2012, preliminary results suggested that IL-1 β was involved in the development of antigen-specific Tfh cells in the airways (70). Since these results, it

has been shown that exposure to IL-1 β in conjunction with ovalbumin leads to significant increases in the levels of specific anti-OVA IgE and IgG (71, 72). Mice that are deficient in the IL-1R1 receptor and sensitized to peanut for 4 weeks showed a large decrease in serum levels of peanut-specific IgE antibodies, as well as anti-peanut IgG1 antibodies. Numbers of Tfh and GC B cells were also dramatically decreased in IL-1R1 $^{-/-}$ mice compared to wild-type mice (73). The role of IL-1 in the pathogenesis of allergies was also suggested by studies showing, for instance, that administration of IL-1Ra to pigs reduced IgE production. Finally, in humans, polymorphisms of IL-1-related genes have been associated with susceptibility to allergic rhinitis (74).

LESSONS FROM THERAPEUTIC TRIALS

Different molecules targeting IL-1 have been or are currently being developed. Among them are monoclonal antibodies directed against IL-1 (Canakinumab, Gevokizumab for instance), a human recombinant IL-1Ra (Anakinra) or a soluble decoy receptor (Rilonacept) (75). Despite widespread use of these IL-1 inhibitors in patients with autoimmune disease, very little has been reported concerning a modification of the humoral response. The only possibly relevant findings are of an increased incidence of infection compared with placebo, but no data have been presented that could support a link with regulation of humoral responses (76).

CONCLUSIONS—PERSPECTIVES

The notion that an IL-1 axis might control humoral immune responses by Tfh and Tfr cells is just emerging. Although it was well known that IL-1 has an important role in immune responses that lead to antibody production, this role was mostly assigned to direct stimulation of an innate immune response, which in turn would control the T and B cell response independently of IL-1. The discovery of a peculiar distribution of IL-1 receptors and IL-1 antagonists on Tfh and Tfr cells led us to revisit the role of IL-1 in the control of antibody production. It is now clear that most Tfh cells from the GCs express IL-1R1 and that in a pure *in vitro* system the addition of IL-1 directly stimulates Tfh to produce the two main B-cell activation cytokines. Furthermore, IL-1 alone

expands Tfh *in vivo*. Thus, a direct role of IL-1 in the activation of Tfh cells appears important for antibody production.

The role of Tfr in controlling this regulation is less well documented. Tfr cells express IL-1R2 and IL-1Ra and are thus equipped to interfere with IL-1-mediated activation of Tfh cells. Recent work has localized most Tfr cells around and not inside GCs, which would be compatible with a role in capturing/neutralizing IL-1 before it can act on Tfh cells (27). Further studies, notably assessing mice knockout for the different receptors on specific cell populations should clarify the mechanistic aspects of the IL-1 axis in Tfr/Tfh cell control of antibody production.

Meanwhile, an existing and large body of evidence indicates that targeting the IL-1 pathway should be an important, although so far ignored, therapeutic approach to many autoimmune diseases. It could work not just by reducing inflammation, which can be fueled by the antibody response, but also by directly reducing this antibody response, thus playing both sides for greater efficacy. By reducing inflammation, it can also improve the efficacy of Tregs which suppressive ability is decreased in high inflammatory context (77–80). We believe that these results should stimulate the investigation of the regulation of IL-1 in many experimental models, from autoimmunity and inflammation to allergy. Furthermore, given the availability of many drugs targeting the IL-1 pathway, and acknowledging that our experimental models of diseases do not reflect human settings of diseases well, innovative clinical trials should play a role in further elucidation of the IL-1 pathway and its therapeutic potential.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

P-GR is an Ecole de l'Inserm Liliane Bettencourt doctoral fellow. This work has been supported the LabEx Transimmunom (ANR-11-IDEX-0004-02), ERC-Advanced TRiPoD (322856) and RHU iMAP grants to DK.

REFERENCES

- Dinarello, CA. A clinical perspective of IL-1 β as the gatekeeper of inflammation. *Eur J Immunol.* (2011) 41:1203–17. doi: 10.1002/eji.201141550
- Dinarello Charles A. Interleukin-1 β , interleukin-18, and the interleukin-1 β converting enzyme. *Ann NY Acad Sci.* (2006) 856:1–11. doi: 10.1111/j.1749-6632.1998.tb08307.x
- Beuscher HU, Günther C, Rölinghoff M. IL-1 beta is secreted by activated murine macrophages as biologically inactive precursor. *J Immunol.* (1990) 144:2179–83.
- The Interleukin-1 Family: Back to the Future-ScienceDirect.* Available online at: <https://www.sciencedirect.com/science/article/pii/S1074761313005153>. (Accessed June 9, 2018)
- Mantovani A, Cassatella MA, Costantini C, Jaillon S. Neutrophils in the activation and regulation of innate and adaptive immunity. *Nat Rev Immunol.* (2011) 11:519–31. doi: 10.1038/nri3024
- Sims JE, Smith DE. The IL-1 family: regulators of immunity. *Nat Rev Immunol.* (2010) 10:89–102. doi: 10.1038/nri2691
- Santarlasci V, Cosmi L, Maggi L, Liotta F, Annunziato F. IL-1 and T helper immune responses. *Front Immunol.* (2013) 4:182. doi: 10.3389/fimmu.2013.00182
- Kryczek I, Wei S, Vatan L, Escara-Wilke J, Szegla W, Keller ET, et al. Cutting edge: opposite effects of IL-1 and IL-2 on the regulation of IL-17+ T cell pool IL-1 subverts IL-2-mediated suppression. *J Immunol.* (2007) 179:1423–6. doi: 10.4049/jimmunol.179.3.1423
- Staruch MJ, Wood DD. The adjuvant activity of interleukin 1 *in vivo*. *J Immunol.* (1983) 130:2191–4.
- Weber A, Wasiliew P, Kracht M. Interleukin-1 (IL-1) pathway. *Sci Signal.* (2010) 3:cm1. doi: 10.1126/scisignal.3105cm1
- Latz E, Xiao TS, Stutz A. Activation and regulation of the inflammasomes. *Nat Rev Immunol.* (2013) 13:397–411. doi: 10.1038/nri3452

12. Thornberry NA, Bull HG, Calaycay JR, Chapman KT, Howard AD, Kostura MJ, et al. A novel heterodimeric cysteine protease is required for interleukin-1 beta processing in monocytes. *Nature* (1992) 356:768–74. doi: 10.1038/356768a0
13. Rubartelli A, Cozzolino F, Talio M, Sitia R. A novel secretory pathway for interleukin-1 beta, a protein lacking a signal sequence. *EMBO J.* (1990) 9:1503–10. doi: 10.1002/j.1460-2075.1990.tb08268.x
14. Lopez-Castejon G, Brough D. Understanding the mechanism of IL-1 β secretion. *Cytokine Growth Factor Rev.* (2011) 22:189–95. doi: 10.1016/j.cytogfr.2011.10.001
15. Cohen P. The TLR and IL-1 signalling network at a glance. *J Cell Sci.* (2014) 127:2383–90. doi: 10.1242/jcs.149831
16. Kawai T, Akira S. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. *Nat Immunol.* (2010) 11:373–84. doi: 10.1038/ni.1863
17. Karin M, Ben-Neriah Y. Phosphorylation meets ubiquitination: the control of NF- κ B activity. *Annu Rev Immunol.* (2000) 18:621–63. doi: 10.1146/annurev.immunol.18.1.621
18. Peters VA, Joesting JJ, Freund GG. IL-1 receptor 2 (IL-1R2) and its role in immune regulation. *Brain Behav Immun.* (2013) 32:1–8. doi: 10.1016/j.bbi.2012.11.006
19. Molgora M, Supino D, Mantovani A, Garlanda C. Tuning inflammation and immunity by the negative regulators IL-1R2 and IL-1R8. *Immunol Rev.* (2018) 281:233–47. doi: 10.1111/immr.12609
20. Seckinger P, Lowenthal JW, Williamson K, Dayer JM, MacDonald HR. A urine inhibitor of interleukin 1 activity that blocks ligand binding. *J Immunol.* (1987) 139:1546–9.
21. Volarevic V, Al-Qahtani A, Arsenijevic N, Pajovic S, Lukic ML. Interleukin-1 receptor antagonist (IL-1Ra) and IL-1Ra producing mesenchymal stem cells as modulators of diabetogenesis. *Autoimmunity* (2010) 43:255–63. doi: 10.3109/08916930903305641
22. Crotty S. T follicular helper cell differentiation, function, and roles in disease. *Immunity* (2014) 41:529–42. doi: 10.1016/j.immuni.2014.10.004
23. Wing JB, Tekgüc M, Sakaguchi S. Control of germinal center responses by T-follicular regulatory cells. *Front Immunol.* (2018) 9:1910. doi: 10.3389/fimmu.2018.01910
24. Chung Y, Tanaka S, Chu F, Nurieva R, Martinez GJ, Rawal S, et al. Follicular regulatory T (Tfr) cells with dual Foxp3 and Bcl6 expression suppress germinal center reactions. *Nat Med.* (2011) 17:983–8. doi: 10.1038/nm.2426
25. Linterman MA, Pierson W, Lee SK, Kallies A, Kawamoto S, Rayner TF, et al. Foxp3+ follicular regulatory T cells control T follicular helper cells and the germinal center response. *Nat Med.* (2011) 17:975–82. doi: 10.1038/nm.2425
26. Wollenberg I, Agua-Doce A, Hernández A, Almeida C, Oliveira VG, Faro J, et al. Regulation of the germinal center reaction by Foxp3+ follicular regulatory T cells. *J Immunol.* (2011) 187:4553–60. doi: 10.4049/jimmunol.1101328
27. Aloulou M, Carr EJ, Gador M, Bignon A, Liblau RS, Fazilleau N, et al. Follicular regulatory T cells can be specific for the immunizing antigen and derive from naive T cells. *Nat Commun.* (2016) 7:10579. doi: 10.1038/ncomms10579
28. Sayin I, Radtke AJ, Vella LA, Jin W, Wherry EJ, Buggert M, et al. Spatial distribution and function of T follicular regulatory cells in human lymph nodes. *J Exp Med.* (2018) 215:1531–42. doi: 10.1084/jem.20171940
29. Ritvo PG, Churlaud G, Quiniou V, Florez L, Brimaud F, Fourcade G, et al. Tfr cells lack IL-2R α but express decoy IL-1R2 and IL-1Ra and suppress the IL-1-dependent activation of Tfh cells. *Sci Immunol.* (2017) 2:eaan0368. doi: 10.1126/sciimmunol.aan0368
30. Botta D, Fuller MJ, Marquez-Lago TT, Bachus H, Bradley JE, Weinmann AS, et al. Dynamic regulation of T follicular regulatory cell responses by interleukin 2 during influenza infection. *Nat Immunol.* (2017) 18:1249–60. doi: 10.1038/ni.3837
31. Wing JB, Kitagawa Y, Locci M, Hume H, Tay C, Morita T, et al. A distinct subpopulation of CD25- T-follicular regulatory cells localizes in the germinal centers. *Proc Natl Acad Sci USA.* (2017) 114:E6400–9. doi: 10.1073/pnas.1705551114
32. Avery DT, Bryant VL, Ma CS, de Waal Malefyt R, Tangye SG. IL-21-induced isotype switching to IgG and IgA by human naive B cells is differentially regulated by IL-4. *J Immunol.* (2008) 181:1767–79. doi: 10.4049/jimmunol.181.3.1767
33. Staats HF, Ennis FA. IL-1 is an effective adjuvant for mucosal and systemic immune responses when coadministered with protein immunogens. *J Immunol.* (1999) 162:6141–7.
34. Li H, Nookala S, Re F. Aluminum hydroxide adjuvants activate caspase-1 and induce IL-1 β and IL-18 release. *J Immunol.* (2007) 178:5271–6. doi: 10.4049/jimmunol.178.8.5271
35. Bromander A, Holmgren J, Lycke N. Cholera toxin stimulates IL-1 production and enhances antigen presentation by macrophages *in vitro*. *J Immunol.* (1991) 146:2908–14.
36. Nakae S, Asano M, Horai R, Iwakura Y. Interleukin-1 beta, but not interleukin-1 alpha, is required for T-cell-dependent antibody production. *Immunology* (2001) 104:402–9. doi: 10.1046/j.1365-2567.2001.01337.x
37. Nakae S, Asano M, Horai R, Sakaguchi N, Iwakura Y. IL-1 enhances T cell-dependent antibody production through induction of CD40 ligand and OX40 on T cells. *J Immunol.* (2001) 167:90–7. doi: 10.4049/jimmunol.167.1.90
38. Nencioni L, Villa L, Tagliabue A, Antoni G, Presentini R, Perin F, et al. *In vivo* immunostimulating activity of the 163-171 peptide of human IL-1 beta. *J Immunol.* (1987) 139:800–4.
39. Reed SG, Pihl DL, Conlon PJ, Grabstein KH. IL-1 as adjuvant. Role of T cells in the augmentation of specific antibody production by recombinant human IL-1 alpha. *J Immunol.* (1989) 142:3129–33.
40. Li H, Pauza CD. CD25+Bcl6low T follicular helper cells provide help to maturing B cells in germinal centers of human tonsil. *Eur J Immunol.* (2015) 45:298–308. doi: 10.1002/eji.201444911
41. Yang H, Tüzün E, Alagappan D, Yu X, Scott BG, Ischenko A, et al. IL-1 receptor antagonist-mediated therapeutic effect in murine myasthenia gravis is associated with suppressed serum proinflammatory cytokines, C3, and anti-acetylcholine receptor IgG1. *J Immunol.* (2005) 175:2018–25. doi: 10.4049/jimmunol.175.3.2018
42. Huang D, Pirskanen R, Hjelmström P, Lefvert AK. Polymorphisms in IL-1beta and IL-1 receptor antagonist genes are associated with myasthenia gravis. *J Neuroimmunol.* (1998) 81:76–81. doi: 10.1016/S0165-5728(97)00161-6
43. Wang CC, Zhang M, Li H, Li XL, Yue LT, Zhang P, et al. Caspase-1 inhibitor regulates humoral responses in experimental autoimmune myasthenia gravis via IL-6- dependent inhibition of STAT3. *Neurosci Lett.* (2017) 656:169–76. doi: 10.1016/j.neulet.2017.05.040
44. McGonagle D, McDermott MF. A proposed classification of the immunological diseases. *PLoS Med.* (2006) 3:e297. doi: 10.1371/journal.pmed.0030297
45. Yoo WS, Chung HK. Recent advances in autoimmune thyroid diseases. *Endocrinol Metab.* (2016) 31:379–85. doi: 10.3803/EnM.2016.31.3.379
46. Zhu C, Ma J, Liu Y, Tong J, Tian J, Chen J, et al. Increased frequency of follicular helper T cells in patients with autoimmune thyroid disease. *J Clin Endocrinol Metab.* (2012) 97:943–50. doi: 10.1210/jc.2011-2003
47. Shehjar F, Afroz D, Misgar RA, Malik SA, Laway BA. Association of polymorphic variants of IL-1 β and IL-1RN genes in the development of Graves' disease in Kashmiri population (North India). *Hum Immunol.* (2018) 79:228–32. doi: 10.1016/j.humimm.2018.02.006
48. Sun L, Zhang X, Dai F, Shen J, Ren C, Zuo C, et al. Elevated interleukin-1 β in peripheral blood mononuclear cells contributes to the pathogenesis of autoimmune thyroid diseases, especially of Hashimoto thyroiditis. *Endocr Res.* (2016) 41:185–92. doi: 10.3109/07435800.2015.1124439
49. Johnsen AK, Plenge RM, Butty V, Campbell C, Dieguez-Gonzalez R, Gomez-Reino JJ, et al. A broad analysis of IL1 polymorphism and rheumatoid arthritis. *Arthritis Rheum.* (2008) 58:1947–57. doi: 10.1002/art.23592
50. Voronov E, Dayan M, Zinger H, Gayvoronsky L, Lin JP, Iwakura Y, et al. IL-1 beta-deficient mice are resistant to induction of experimental SLE. *Eur Cytokine Netw.* (2006) 17:109–16.
51. Lee J, Rhee MH, Kim E, Cho JY. BAY 11-7082 is a broad-spectrum inhibitor with anti-inflammatory activity against multiple targets. *Mediators Inflamm.* (2012) 2012:416036. doi: 10.1155/2012/416036
52. Zhao J, Zhang H, Huang Y, Wang H, Wang S, Zhao C, et al. Bay11-7082 attenuates murine lupus nephritis via inhibiting NLRP3 inflammasome and NF- κ B activation. *Int Immunopharmacol.* (2013) 17:116–22. doi: 10.1016/j.intimp.2013.05.027

53. Kahlenberg JM, Yalavarthi S, Zhao W, Hodgin JB, Reed TJ, Tsuji NM, et al. An essential role of caspase 1 in the induction of murine lupus and its associated vascular damage. *Arthritis Rheumatol.* (2014) 66:152–62. doi: 10.1002/art.38225
54. Moosig F, Zeuner R, Renk C, Schröder JO. IL-1RA in refractory systemic lupus erythematosus. *Lupus* (2004) 13:605–6. doi: 10.1191/0961203304lu1047cr
55. Ostendorf B, Iking-Konert C, Kurz K, Jung G, Sander O, Schneider M. Preliminary results of safety and efficacy of the interleukin 1 receptor antagonist anakinra in patients with severe lupus arthritis. *Ann Rheum Dis.* (2005) 64:630–3. doi: 10.1136/ard.2004.025858
56. Tayer-Shifman OE, Ben-Chetrit E. Refractory macrophage activation syndrome in a patient with SLE and APLA syndrome-successful use of PET-CT and Anakinra in its diagnosis and treatment. *Mod Rheumatol.* (2015) 25:954–7. doi: 10.3109/14397595.2013.844403
57. Elliott C, Lindner M, Arthur A, Brennan K, Jarius S, Hussey J, et al. Functional identification of pathogenic autoantibody responses in patients with multiple sclerosis. *Brain J Neurol.* (2012) 135:1819–33. doi: 10.1093/brain/awsl05
58. Kanter JL, Narayana S, Ho PP, Catz I, Warren KG, Sobel RA, et al. Lipid microarrays identify key mediators of autoimmune brain inflammation. *Nat Med.* (2006) 12:138–43. doi: 10.1038/nm1344
59. O'Connor KC, Appel H, Bregoli L, Call ME, Catz I, Chan JA, et al. Antibodies from inflamed central nervous system tissue recognize myelin oligodendrocyte glycoprotein. *J Immunol.* (2005) 175:1974–82. doi: 10.4049/jimmunol.175.3.1974
60. O'Connor KC, Chitnis T, Griffin DE, Piyasirisilp S, Bar-Or A, Khoury S, et al. Myelin basic protein-reactive autoantibodies in the serum and cerebrospinal fluid of multiple sclerosis patients are characterized by low-affinity interactions. *J Neuroimmunol.* (2003) 136:140–8. doi: 10.1016/S0165-5728(03)00002-X
61. Wu GF, Alvarez E. The immuno-pathophysiology of multiple sclerosis. *Neuro Clin.* (2011) 29:257–78. doi: 10.1016/j.ncl.2010.12.009
62. Hauser SL, Waubant E, Arnold DL, Vollmer T, Antel J, Fox RJ, et al. B-cell depletion with rituximab in relapsing-remitting multiple sclerosis. *N Engl J Med.* (2008) 358:676–88. doi: 10.1056/NEJMoa0706383
63. Keegan M, König F, McClelland R, Brück W, Morales Y, Bitsch A, et al. Relation between humoral pathological changes in multiple sclerosis and response to therapeutic plasma exchange. *Lancet* (2005) 366:579–82. doi: 10.1016/S0140-6736(05)67102-4
64. Lin C-C, Edelson BT. New insights into the role of IL-1 β in experimental autoimmune encephalomyelitis and multiple sclerosis. *J Immunol.* (2017) 198:4553–60. doi: 10.4049/jimmunol.1700263
65. Schiffenbauer J, Streit WJ, Butfiloski E, LaBow M, Edwards C, Moldawer LL. The induction of EAE is only partially dependent on TNF receptor signaling but requires the IL-1 type I receptor. *Clin Immunol.* (2000) 95:117–23. doi: 10.1006/clim.2000.4851
66. Martin D, Near SL. Protective effect of the interleukin-1 receptor antagonist (IL-1ra) on experimental allergic encephalomyelitis in rats. *J Neuroimmunol.* (1995) 61:241–5. doi: 10.1016/0165-5728(95)00108-E
67. Cherniavsky AC, Páez MC, Periolo N, Correa P, Guillén L, Niveloni SI, et al. The simultaneous presence of IL-1B and TNFA two-positions risk haplotypes enhances the susceptibility for celiac disease. *Cytokine* (2008) 42:48–54. doi: 10.1016/j.cyto.2008.01.015
68. Palová-Jelínková L, Dánová K, Drašarová H, Dvůrák M, Funda DP, Fundová P, et al. Pepsin digest of wheat gliadin fraction increases production of IL-1 β via TLR4/MyD88/TRIF/MAPK/NF- κ B signaling pathway and an NLRP3 inflammasome activation. *PLoS ONE* (2013) 8:e62426. doi: 10.1371/journal.pone.0062426
69. Ritvo PG, Saadawi A, Barennes P, Quiniou V, Chaara W, El Soufi K, et al. High-resolution repertoire analysis reveals a major bystander activation of Tfh and Tfr cells. *Proc Natl Acad Sci USA* (2018) 115:9604–9. doi: 10.1073/pnas.1808594115
70. Kobayashi T, Iijima K, Kita H. Exposure to IL-1-family cytokines regulates differential development of antigen-specific Th2 cells and T follicular helper (Tfh) cells in the airway. *J Allergy Clin Immunol.* (2012) 129:AB145. doi: 10.1016/j.jaci.2011.12.487
71. Kobayashi T, Iijima K, Dent AL, Kita H. Follicular helper T (Tfh) cells mediate IgE antibody response to airborne allergens. *J Allergy Clin Immunol.* (2017) 139:300–313.e7. doi: 10.1016/j.jaci.2016.04.021
72. Kobayashi T, Iijima K, Checkel JL, Kita H. IL-1 family cytokines drive Th2 and Th17 cells to innocuous airborne antigens. *Am J Respir Cell Mol Biol.* (2013) 49:989–98. doi: 10.1165/rcmb.2012-0444OC
73. Dolence JJ, Kobayashi T, Iijima K, Krempski J, Drake LY, Dent AL, et al. Airway exposure initiates peanut allergy by involving the IL-1 pathway and T follicular helper cells in mice. *J Allergy Clin Immunol.* (2018) 142:1145–58. doi: 10.1016/j.jaci.2017.11.020
74. Nambu A, Nakae S. IL-1 and allergy. *Allergol Int.* 59:125–35 (2010). doi: 10.2332/allergolint.10-RAI-0190
75. Dinarello CA, Simon A, van der Meer JWM. Treating inflammation by blocking interleukin-1 in a broad spectrum of diseases. *Nat Rev Drug Discov.* (2012) 11:633–52. doi: 10.1038/nrd3800
76. Winthrop KL, Mariette X, Silva JT, Benamu E, Calabrese LH, Dumusc A, et al. ESCMID Study Group for Infections in Compromised Hosts (ESGICH) consensus document on the safety of targeted and biological therapies: an infectious diseases perspective (Soluble immune effector molecules [II]: agents targeting interleukins, immunoglobulins and complement factors). *Clin Microbiol Infect.* (2018) 24(Suppl. 2):S21–40. doi: 10.1016/j.cmi.2018.02.002
77. Billiard F, Litvinova E, Saadoun D, Djelti F, Klatzmann D, Cohen JL, et al. Regulatory and effector T cell activation levels are prime determinants of *in vivo* immune regulation. *J Immunol.* (2006) 177:2167–74. doi: 10.4049/jimmunol.177.4.2167
78. Klatzmann D, Abbas AK. The promise of low-dose interleukin-2 therapy for autoimmune and inflammatory diseases. *Nat Rev Immunol.* (2015) 15:283–94. doi: 10.1038/nri3823
79. Li Z, Lin F, Zhuo C, Deng G, Chen Z, Yin S, et al. PIM1 kinase phosphorylates the human transcription factor FOXP3 at serine 422 to negatively regulate its activity under inflammation. *J Biol Chem.* (2014) 289:26872–81. doi: 10.1074/jbc.M114.586651
80. Yamaguchi T, Wing JB, Sakaguchi S. Two modes of immune suppression by Foxp3(+) regulatory T cells under inflammatory or non-inflammatory conditions. *Semin Immunol.* (2011) 23:424–30. doi: 10.1016/j.smim.2011.10.002

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2019 Ritvo and Klatzmann. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



P2RX7 Deletion in T Cells Promotes Autoimmune Arthritis by Unleashing the Tfh Cell Response

Krysta M. Felix^{1†}, Fei Teng^{1†}, Nicholas A. Bates^{1†}, Heqing Ma¹, Ivan A. Jaimez¹, Kiah C. Sleiman¹, Nhan L. Tran¹ and Hsin-Jung Joyce Wu^{1,2*}

¹ Department of Immunobiology, University of Arizona, Tucson, AZ, United States, ² Arizona Arthritis Center, College of Medicine, University of Arizona, Tucson, AZ, United States

OPEN ACCESS

Edited by:

Maria Pia Cicalese,
San Raffaele Scientific Institute
(IRCCS), Italy

Reviewed by:

Hu Zeng,
Mayo Clinic, United States
Hui Xiao,
Institut Pasteur of Shanghai (CAS),
China

*Correspondence:

Hsin-Jung Joyce Wu
joycewu@email.arizona.edu

[†]These authors have contributed
equally to this work

Specialty section:

This article was submitted to
T Cell Biology,
a section of the journal
Frontiers in Immunology

Received: 10 July 2018

Accepted: 15 February 2019

Published: 19 March 2019

Citation:

Felix KM, Teng F, Bates NA, Ma H, Jaimez IA, Sleiman KC, Tran NL and Wu H-JJ (2019) P2RX7 Deletion in T Cells Promotes Autoimmune Arthritis by Unleashing the Tfh Cell Response. *Front. Immunol.* 10:411. doi: 10.3389/fimmu.2019.00411

Rheumatoid arthritis (RA) is an autoimmune disease that affects ~1% of the world's population. B cells and autoantibodies play an important role in the pathogenesis of RA. The P2RX7 receptor is an ATP-gated cation channel and its activation results in the release of pro-inflammatory molecules. Thus, antagonists of P2RX7 have been considered to have potential as novel anti-inflammatory therapies. Although originally identified for its role in innate immunity, P2RX7 has recently been found to negatively control Peyer's patches (PP) T follicular helper cells (Tfh), which specialize in helping B cells, under homeostatic conditions. We have previously demonstrated that PP Tfh cells are required for the augmentation of autoimmune arthritis mediated by gut commensal segmented filamentous bacteria (SFB). Thus, we hypothesized that P2RX7 is required to control autoimmune disease by keeping the Tfh cell response in check. To test our hypothesis, we analyzed the impact of P2RX7 deficiency *in vivo* using both the original K/BxN autoimmune arthritis model and T cell transfers in the K/BxN system. We also examined the impact of P2RX7 ablation on autoimmune development in the presence of the gut microbiota SFB. Our data illustrate that contrary to exerting an anti-inflammatory effect, P2RX7 deficiency actually enhances autoimmune arthritis. Interestingly, SFB colonization can negate the difference in disease severity between WT and P2RX7-deficient mice. We further demonstrated that P2RX7 ablation in the absence of SFB caused reduced apoptotic Tfh cells and enhanced the Tfh response, leading to an increase in autoantibody production. It has been shown that activation of TIGIT, a well-known T cell exhaustion marker, up-regulates anti-apoptotic molecules and promotes T cell survival. We demonstrated that the reduced apoptotic phenotype of *P2rx7*^{-/-} Tfh cells is associated with their increased expression of TIGIT. This suggested that while P2RX7 was regulating the Tfh population by promoting cell death, TIGIT may have been opposing P2RX7 by inhibiting cell death. Together, these results demonstrated that systemic administration of general P2RX7 antagonists may have detrimental effects in autoimmune therapies, especially in Tfh cell-dependent autoimmune diseases, and cell-specific targeting of P2RX7 should be considered in order to achieve efficacy for P2RX7-related therapy.

Keywords: P2RX7, autoimmune, microbiota, apoptosis, TIGIT

INTRODUCTION

Rheumatoid arthritis (RA) is an autoimmune disease that causes chronic inflammation of the joints and affects ~1% of the world's population. Genetics play an important role in RA and the disease concordance rate between monozygotic twins with RA is ~15%, (1, 2). This suggests that other factors are also critically involved in RA pathogenesis, including smoking, gender, and a more recently discovered factor, the composition of the gut microbiota (3–6). Autoantibodies (auto-Abs) play critical roles in the pathogenesis of RA as they contribute to immune complex formation and complement activation, leading to tissue damage in the joints (7–12). T follicular helper (Tfh) cells are a subset of CD4⁺ T cells that co-expresses high levels of the inhibitory co-receptor PD-1 and the chemokine receptor CXCR5 (13–15). The function of Tfh cells is to help germinal center B cells produce high-titer, high-affinity, isotype-switched antibodies (Abs) and differentiate into long-lived plasma cells. Therefore, an excessive Tfh cell response can lead to over-productive auto-Ab responses and autoimmune conditions including RA (16).

High concentrations of extracellular ATP, such as those released by dying cells, can act as a danger signal by binding to the P2RX7 purinergic receptor, an ATP-gated cation channel (17). This activates the NLRP3 inflammasome pathway, which ultimately leads to the maturation and release of the pro-inflammatory cytokines IL-1 β and IL-18 (18). Therefore, P2RX7 has been targeted as a means of developing anti-inflammatory therapies in many autoimmune diseases (19). However, studies examining the function of P2RX7 in autoimmunity using P2RX7 deficient mouse models suggest a complicated role for P2RX7, as P2RX7 deficiency can either suppress or exaggerate disease phenotypes (20–22). In the context of rheumatoid arthritis, there have been several clinical trials using P2RX7 antagonists as a means of reducing inflammation (19, 23, 24). However, while early results appeared to support the beneficial effects of such treatment, P2RX7 antagonists failed to improve arthritic symptoms in two recent RA clinical trials (23, 24). Understanding the mechanisms by which P2RX7 impacts autoimmunity will provide the knowledge required to properly target P2RX7 for therapeutic purposes. This is an urgent task, since despite some initial setbacks, enthusiasm for P2RX7 antagonist therapy remains strong, as evidenced by the numerous ongoing clinical trials with new P2RX7 agonist and antagonist compounds (17, 19, 25).

Interestingly, although originally identified for its crucial role in the innate immune response (19), P2RX7 has recently been found to also play key roles in regulating T cell populations. A pioneer report has shown that P2RX7 activation negatively controls Tfh cell numbers in Peyer's Patches (PPs), a type of gut-associated lymphoid tissue, to promote host-microbiota mutualism under homeostatic conditions (26). Additionally, P2RX7 drives Th1 cell differentiation and controls the follicular helper T cell population to protect against *Plasmodium chabaudi* malaria (27). However, the role of P2RX7 in the Tfh cell response under autoimmune conditions is not known. Importantly, with regard to inflammatory arthritis, a study found that 2 of 9 patients with systemic juvenile idiopathic arthritis had loss-of-function

variants in *P2rx7* (28). Therefore, we hypothesized that P2RX7 deficiency enhances autoimmune disease by increasing the Tfh cell response. We have previously demonstrated that the gut microbiota constituent segmented filamentous bacteria (SFB) promote autoimmune arthritis via inducing PP Tfh cells (29). Therefore, we also examined the impact of P2RX7 ablation on autoimmune development in the presence of gut microbiota SFB.

Here, we use the K/BxN [KRN T cell receptor (TCR) transgenic mice on the C57/BL6 (B6) background x NOD] model to test our hypothesis. The K/BxN model is a murine autoimmune arthritis model in which KRN T cells recognize glucose-6-phosphate isomerase (GPI), the self-antigen presented by MHC class II I-A^{g7} from NOD mice (30). These activated T cells can in turn activate B cells to produce anti-GPI auto-Abs. K/BxN mice share many clinical and histologic features with human RA patients (31). As in many human autoimmune diseases including RA, auto-Abs play important pathological roles in K/BxN disease development (31). An advantage of the K/BxN model is that it has an easily distinguishable initial T-B cell interaction phase and a later effector phase involving innate immune players that allows for a straightforward analysis of the immune response (32–34). Thus, the intrinsic role of T cells can be easily dissected out by using the K/BxN T cell transfer model. This is done by transferring K/BxN T cells into T cell-deficient mice that express MHC II I-A^{g7} (30, 35). This approach allows for the examination of T cell-specific P2RX7 contributions and avoids many confounding effects from genetic modification of whole animals. Here we demonstrated that P2RX7 deficiency in the whole mouse caused augmented autoimmune arthritis, but SFB colonization does not further exacerbate disease in P2RX7-deficient K/BxN mice, as it does in wild type (WT) K/BxN mice. Interestingly, the arthritis enhancement in SFB(–) mice was reproducible simply by deleting P2RX7 in T cells, which led to an enhanced Tfh cell response. Thus, unlike the anti-inflammatory effect of P2RX7 blockade in innate immunity reported previously, our results indicated that P2RX7 deletion in T cells actually enhances autoimmunity by unleashing the Tfh cell response.

MATERIALS AND METHODS

Mice

KRN TCR transgenic mice in the C57BL/6 (B6) background (KRN), TCR $\alpha^{-/-}$.B6, and TCR $\alpha^{-/-}$.NOD mice were originally obtained from the mouse colony of Drs. Diane Mathis and Christophe Benoist at the Jackson Laboratory (Jax). K/BxN mice were generated by crossing KRN mice to NOD mice (All K/BxN experimental mice are the F1 offspring of KRN and NOD parents). *P2rx7*^{-/-}.B6 and *P2rx7*^{-/-}.NOD mice were purchased from Jax. *P2rx7*^{-/-}.B6 were crossed to KRN mice to generate *P2rx7*^{-/-}.KRN mice. *P2rx7*^{-/-}.K/BxN mice were generated by crossing *P2rx7*^{-/-}.KRN (in B6 background) to *P2rx7*^{-/-}.NOD mice. TCR $\alpha^{-/-}$.BxN mice were generated by crossing TCR $\alpha^{-/-}$.B6 with TCR $\alpha^{-/-}$.NOD mice. TCR $\alpha^{-/-}$.BxN mice thus bear the NOD MHC class II, I-A^{g7}, for self-Ag presentation. Age- and gender-matched K/BxN

mice between 5 and 7 weeks old were used in experiments. The K/BxN autoimmune model is a T cell receptor transgenic model, therefore, its disease development is robust and occurs in 100% of the F1 (KRNxNOD) offspring, regardless of gender (32). Additionally, we compared arthritis development between male and female K/BxN mice and found no significant difference in disease severity (**Supplementary Figure 1**). For transfer experiments, mice were given transfer cells at 6 weeks old, and analysis was performed 2 weeks after transfer. Ankle thickness was measured with a caliper (J15 Blet micrometer) as described previously (36). All mice were housed at the SPF animal facility at the University of Arizona. All experiments were conducted in accordance with the guidelines of the University of Arizona Institutional Animal Care and Use Committee Protocol number 11-278.

Antibodies and Flow Cytometry

For surface staining, fluorophore-conjugated mAbs specific for CD4 (RM4-5), CD19 (6D5), CD25 (PC61), CD11c (N418), CD11b (M1/70), Gr-1 (RB6-8C5), CD45 (30-F11), TCR β (H57-597), PD-1 (RMP1-30), CD8 α (53-6.7), CD3 (17A2), and TIGIT (1G9) were obtained from BioLegend. Abs recognizing CXCR5 (2G8) were from BD Pharmingen. Abs recognizing P2RX7 (Hano43) were from Novus Biologicals. For intranuclear staining, buffers from a Foxp3 Staining Buffer Set (eBioscience) were used to stain with Abs recognizing Bcl-6(K112-91, BD Pharmingen) and Foxp3 (FJK-16s, eBioscience). For the cell death assay, cells were stained with Live/Dead[®] Yellow Dye (LifeTechnologies) and then surface markers, and Annexin V binding accomplished using the Annexin V/Dead Cell Apoptosis Kit (LifeTechnologies) according to the manufacturer's instructions. Cells were run on an LSRII (BD Biosciences), and analyses were performed with FlowJo (TreeStar) software.

ELISA

Anti-GPI Ab titers were measured as described (36). Briefly, ELISA plates were coated with recombinant mouse GPI at 5 μ g/ml, and diluted mouse sera were added. Subsequently, plates were washed and alkaline-phosphatase (AP)-conjugated anti-mouse IgG Abs (Jackson ImmunoResearch) were added. After the final wash, AP substrate was added and titers were quantified as optical density values via an ELISA reader. Ab titers were expressed as arbitrary units, which were calculated from serial dilutions of sample serum and defined as the reciprocal of the highest dilution that gave an above background O.D. value set as 0.15.

Cell Transfer and Serum Transfer

T cells were transferred as described previously (35). Briefly, splenic CD4⁺ T cells (5x10⁵) were enriched by CD4-conjugated MACS beads from K/BxN or *P2rx7*^{-/-}.K/BxN mice and adoptively transferred by retro-orbital (r.o.) injection into 6 week old SFB(-) or SFB(+) *Tcra*^{-/-}.BxN recipients. SFB status was manipulated by gavaging recipient mice with SFB prior to cell transfer. At the indicated time point after transfer, tissues from recipient mice were harvested for flow cytometry analysis and sera were collected for ELISA.

Microbiota Reconstitution and Quantification

Our SPF mouse colony was derived as previously described (29). SFB(-) *Tcra*^{-/-}.BxN mice were weaned at 21 days old and rested for 1 day. Then, mice were orally gavaged for 3 consecutive days starting at 22 days old. SFB(-) mice were the ungavaged littermate controls. The SFB colonization status was examined 10 days after the first gavage by SFB-specific 16S rRNA quantitative PCR (36). To determine the change in SFB induced by transfer of *P2rx7*^{-/-}.K/BxN T cells, fecal samples were also collected at the time of analysis, 2 weeks after the T cell transfer, and SFB colonization was examined by quantitative PCR.

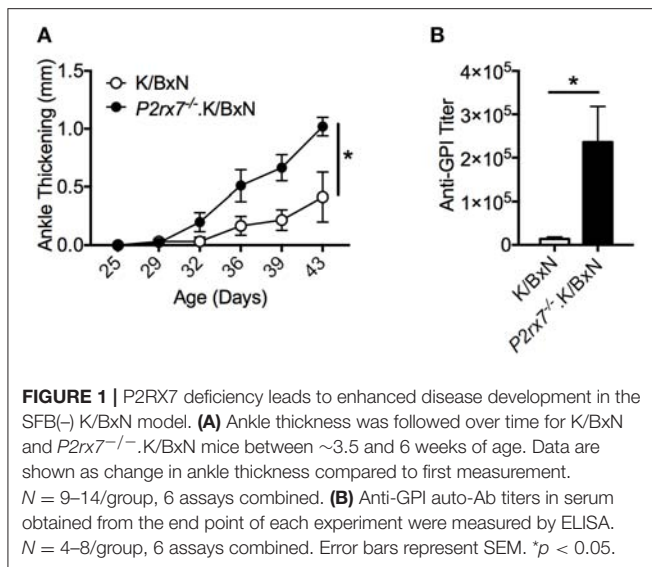
Statistical Analysis

Asterisks indicate statistical significance. Differences were considered significant when $P < 0.05$ by Student's *t*-test (two-tailed, unpaired, with Welch's correction) or one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons. To compare ankle thickening, the area under the curve (AUC) was calculated for each mouse within an experimental set followed by a Student's *t*-test between the groups (Prism 6, Graph-Pad Software). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

RESULTS

P2RX7 Deficiency Enhances Autoimmune Arthritis Development

We first determined the role of P2RX7 in the spontaneous K/BxN autoimmune arthritis model. Genetic P2RX7 deletion (*P2rx7*^{-/-} mice) in other autoimmune models has generated results ranging from suppression, to no change, to exacerbation of the autoimmune response compared to WT controls (20–22). It is worth mentioning that some of these studies were not using fully backcrossed mice and alternative P2RX7 functions due to allelic differences among common inbred strains have been reported (37–40). Due to the complexity of P2RX7, the WT K/BxN and *P2rx7*^{-/-}.K/BxN used in this study were in a fixed B6xNOD background. We found enhanced arthritis development in *P2rx7*^{-/-} compared to WT K/BxN mice at an early age, 4–6 weeks old (**Figure 1A**). In the K/BxN autoimmune arthritis model, disease development is driven largely by anti-GPI auto-Abs (30). Therefore, we examined the anti-GPI auto-Ab titer in *P2rx7*^{-/-} compared to WT K/BxN mice. The increased susceptibility to disease development in *P2rx7*^{-/-} mice corresponded with an increased auto-Ab response (**Figure 1B**). In the K/BxN model, therefore, complete P2RX7 deficiency promoted an enhanced auto-Ab response, provoking early disease onset compared to WT controls. There was no significant change in T cell development in the thymus and we also did not find any difference in peripheral immune populations including CD4⁺ T cells and CD19⁺ B cells in spleen and PPs between K/BxN and *P2rx7*^{-/-}.K/BxN mice (**Table 1**). Furthermore, no difference was found in the innate populations including Gr-1⁺CD11b⁺ neutrophils and CD11c⁺ or CD11b⁺ antigen presenting cells in spleen between K/BxN and *P2rx7*^{-/-}.K/BxN mice (**Table 1**).



Differing Impacts of P2RX7 Deficiency on Systemic and PP Tfh Responses

Next, we aimed to determine the cellular mechanism that caused the increased arthritis in *P2rx7*^{-/-}.K/BxN mice. P2RX7 deficiency has been shown to increase PP Tfh cells in C57BL/6 (B6) mice, leading to an increase in IgA production in the PPs (26). PP Tfh cells also play an important pathological role in the development of arthritis in the K/BxN model, and an increase in PP Tfh cells can lead to Tfh migration to systemic sites, driving auto-Ab production (29). Because of this, we examined whether P2RX7 deficiency causes an enhancement of the Tfh response in both the gut and systemic sites, leading to exacerbation of disease and auto-Ab production in the K/BxN model. As defined in previous publications, we identified Tfh cells by high PD-1 and CXCR5 expression in CD4⁺ T cells (29, 41–45). We observed strong PP Tfh induction in *P2rx7*^{-/-}.K/BxN mice, analogous to what has been shown in P2RX7-deficient C57/BL6 mice [Figure 2A; (26)], however, there was no difference in the number of Tfh cells in the spleen between *P2rx7*^{-/-} and WT K/BxN mice (Figure 2A). Because one of the major consequences of P2RX7 activation is triggering cell death, and P2RX7 controls the PP Tfh population by inducing cell death (26), we examined whether differing levels of cell death in P2RX7-deficient splenic and PP Tfh cells explain the different responses to P2RX7 deletion in these tissues. Consistent with previous reports, *P2rx7*^{-/-}.K/BxN PP Tfh cells had a significant reduction in cell death, as shown by decreased Annexin V staining (among intact cells that excluded Live/Dead Yellow, a membrane-impermeable dye) compared to WT K/BxN Tfh cells (Figure 2B). This method of measuring cell death has been shown to reliably distinguish early apoptotic cells from cells in late-stage cell death (46). However, the difference in percentage of apoptotic splenic Tfh cells between WT and P2RX7 deficient mice is less compared to that of PP Tfh cells (Figure 2B); i.e., the average ratio of WT to *P2rx7*^{-/-}.K/BxN apoptotic Tfh cell

TABLE 1 | Comparison of major cell groups in K/BxN and *P2rx7*^{-/-}.K/BxN mice.

Organ	Cell group	K/BxN	<i>P2rx7</i> ^{-/-} .K/BxN
Thymus	CD8, CD4 DN (% of TCRβ ⁺)	14.57 ± 1.92	13.10 ± 2.14
	CD8, CD4 DP (% of TCRβ ⁺)	76.90 ± 2.86	78.10 ± 3.97
	CD8 SP (% of TCRβ ⁺)	1.15 ± 0.15	1.26 ± 0.34
	CD4 SP (% of TCRβ ⁺)	1.08 ± 0.16	0.88 ± 0.13
	CD4 ⁺ (% of splenocytes)	3.10 ± 0.22	3.47 ± 0.20
Spleen	CD19 ⁺ (% of splenocytes)	73.32 ± 1.15	71.42 ± 1.20
	CD11c ⁺ (% of CD19-TCRβ-Gr-1-)	10.14 ± 1.69	10.08 ± 1.62
	CD11b ⁺ (% of CD19-TCRβ-Gr-1-)	28.05 ± 2.25	30.59 ± 4.22
	Gr-1 ⁺ CD11b ⁺ neutrophils (% of CD19-TCRβ-)	16.69 ± 0.22	21.82 ± 3.47
	CD4 ⁺ (% of splenocytes)	3.11 ± 0.32	3.69 ± 0.35
PPs	CD19 ⁺ (% of splenocytes)	66.48 ± 1.420	59.37 ± 1.861

percentage in the spleen (2.53) is less compared to the PPs (16.37). This indicates that PP Tfh cells are more sensitive to P2RX7-mediated apoptosis than splenic Tfh cells. To determine whether the increase in PP Tfh cells is a Tfh-specific effect, we also examined PD-1^{lo/-}CXCR5^{lo/-} CD4⁺ T cells, a population referred to as “non-Tfh cells,” (29, 41). Our data demonstrated that there is no change in number or frequency of the non-Tfh PP population (Supplementary Figure 2A). While there is a decrease in apoptosis in the P2RX7 deficient non-Tfh population, it is again less than the decrease in apoptosis of the Tfh population (Supplementary Figure 2A vs. Figure 2B). We hypothesized that different expression levels of P2RX7 might explain the differences in Tfh cell induction and Tfh cell death we detected in the spleen compared to the PPs. Indeed, while P2RX7 expression on Tfh cells from both organs exceeded P2RX7 expression on non-Tfh cells, P2RX7 expression was much higher (>2-fold) on PP Tfh cells than splenic Tfh cells (Figure 2C). Differential expression of Bcl-6 could also potentially help explain the increased Tfh population in *P2rx7*^{-/-}.K/BxN compared to WT K/BxN mice. Expression of the transcription factor Bcl-6 in CD4⁺ T cells is both necessary and sufficient to drive CD4⁺ T cell differentiation into Tfh cells, and thus Bcl-6 is considered a master regulator of Tfh differentiation (47–49). However, when we examined Bcl-6 expression in splenic and PP non-Tfh cells, we found no difference in expression levels between *P2rx7*^{-/-} and WT K/BxN mice (Figure 2D). Taken together, these data suggested that P2RX7 controls the Tfh cell population by inducing cell death, however lower P2RX7 expression levels on splenic Tfh cells limit the ability of P2RX7 to control the Tfh cell population in this systemic site.

T Cell-Intrinsic P2RX7 Deficiency Enhances Arthritis Development and Increases the PP Tfh Cell Response

To dissect whether the enhanced Tfh cell response and arthritis severity observed in P2RX7 deficiency of the whole K/BxN mouse is mediated by a T cell-intrinsic effect of P2RX7, we used the K/BxN T cell transfer system (Figure 3A). In this

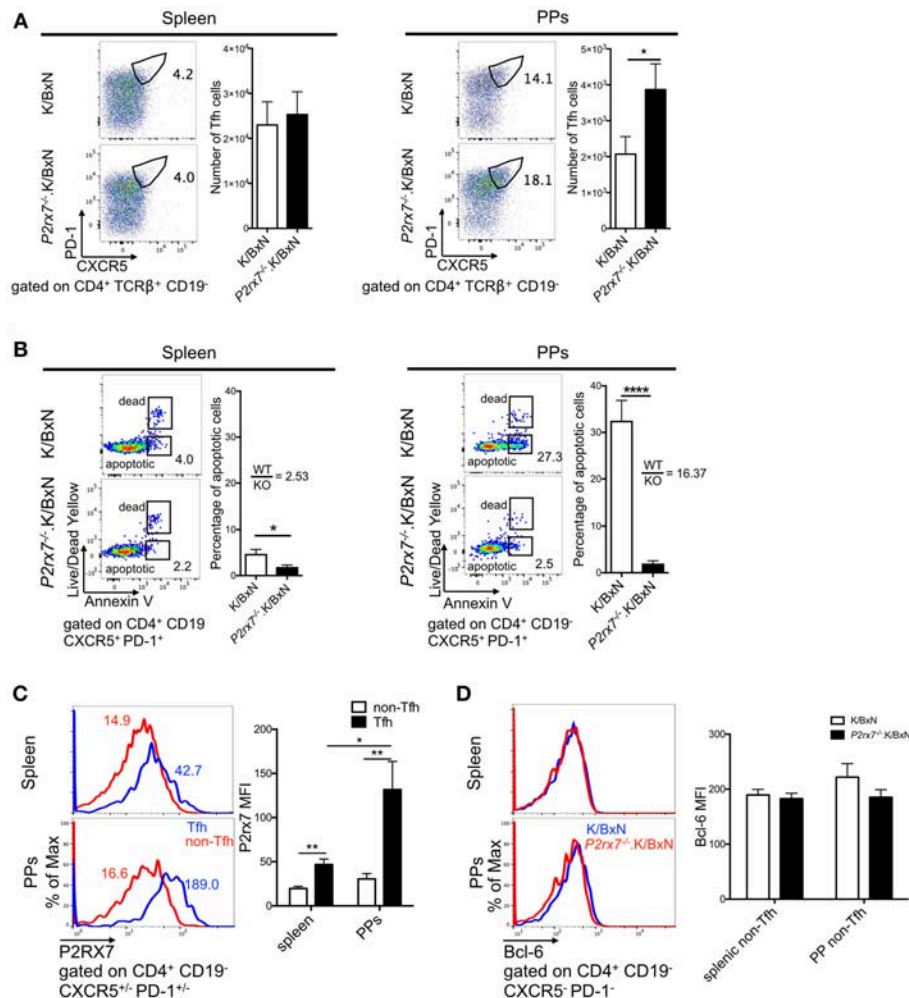


FIGURE 2 | P2RX7 deletion induces the Tfh cell response in PPs but not spleen of the SFB(-) K/BxN mice. **(A)** Splenocytes and PP cells from 5 to 6 week old K/BxN and *P2rx7*^{-/-}.K/BxN mice were stained with Abs against CD4, CD19, PD-1, and CXCR5. Values in the representative plots indicate the percentage of Tfh cells in total CD4⁺ T cells. Compiled graphs of the number of Tfh cells are also shown as mean + SEM. *N* = 17–18/group, 8 independent assays combined. **(B)** Tfh cells from spleen and PPs of K/BxN and *P2rx7*^{-/-}.K/BxN mice were analyzed for Annexin V binding among Live/Dead Yellow⁻ cells. Representative plots and compiled graphs are shown. Ratios calculated by dividing the average of the K/BxN (WT) group by the average of the *P2rx7*^{-/-}.K/BxN (KO) group. *N* = 6–7/group, 4 independent assays combined. **(C)** P2RX7 expression was detected by flow cytometry on non-Tfh and Tfh cells from spleen and PPs of K/BxN and *P2rx7*^{-/-}.K/BxN mice. Representative plots and compiled graphs are shown. *N* = 8–9/group, 3 independent assays combined. **(D)** Bcl-6 expression was detected by flow cytometry on non-Tfh cells from spleen and PPs of K/BxN and *P2rx7*^{-/-}.K/BxN mice. Representative plots and compiled graphs are shown. *N* = 13–17/group, 6 independent assays combined. Error bars represent means + SEM. **p* < 0.05, ***p* < 0.01, ****p* < 0.0001.

system, arthritis is generated by transferring K/BxN T cells into TCR α ^{-/-}.BxN (T cell deficient B6xNOD) mice, which allows the transferred K/BxN T cells to recognize GPI peptide presented on MHC II I-A^{B7} and develop arthritis (30, 50). *P2rx7*^{-/-} or WT K/BxN CD4⁺ T cells were transferred into TCR α ^{-/-}.BxN mice as described previously (29). We found that mice that received *P2rx7*^{-/-}.K/BxN T cells developed worse arthritis and had increased anti-GPI auto-Abs, indicating amplified autoimmunity compared to mice that received WT K/BxN T cells (Figures 3B,C). Additionally, we found that mice receiving *P2rx7*^{-/-}.K/BxN T cells demonstrated a trend toward an increase in splenic Tfh cells, and a significant increase in

PP Tfh cells compared to mice receiving WT K/BxN T cells (Figure 3D). We found no change in non-Tfh cells in PPs between mice that received WT K/BxN or *P2rx7*^{-/-}.K/BxN T cells (Figure 3D, Supplementary Figure 2B). Thus, as in total P2RX7 deletion, our data suggested that T cell-intrinsic P2RX7 deficiency significantly enhanced the Tfh response in the PPs. However, unlike the total P2RX7 deletion, there was a trend toward an increase in splenic Tfh cells in the spleen. Consistent with our data from the whole-mouse P2RX7 knockout system, we also found that Tfh cell apoptosis was significantly inhibited in both the spleen and PPs in mice that received *P2rx7*^{-/-} T cells compared to those that received WT T cells (Figure 3E).

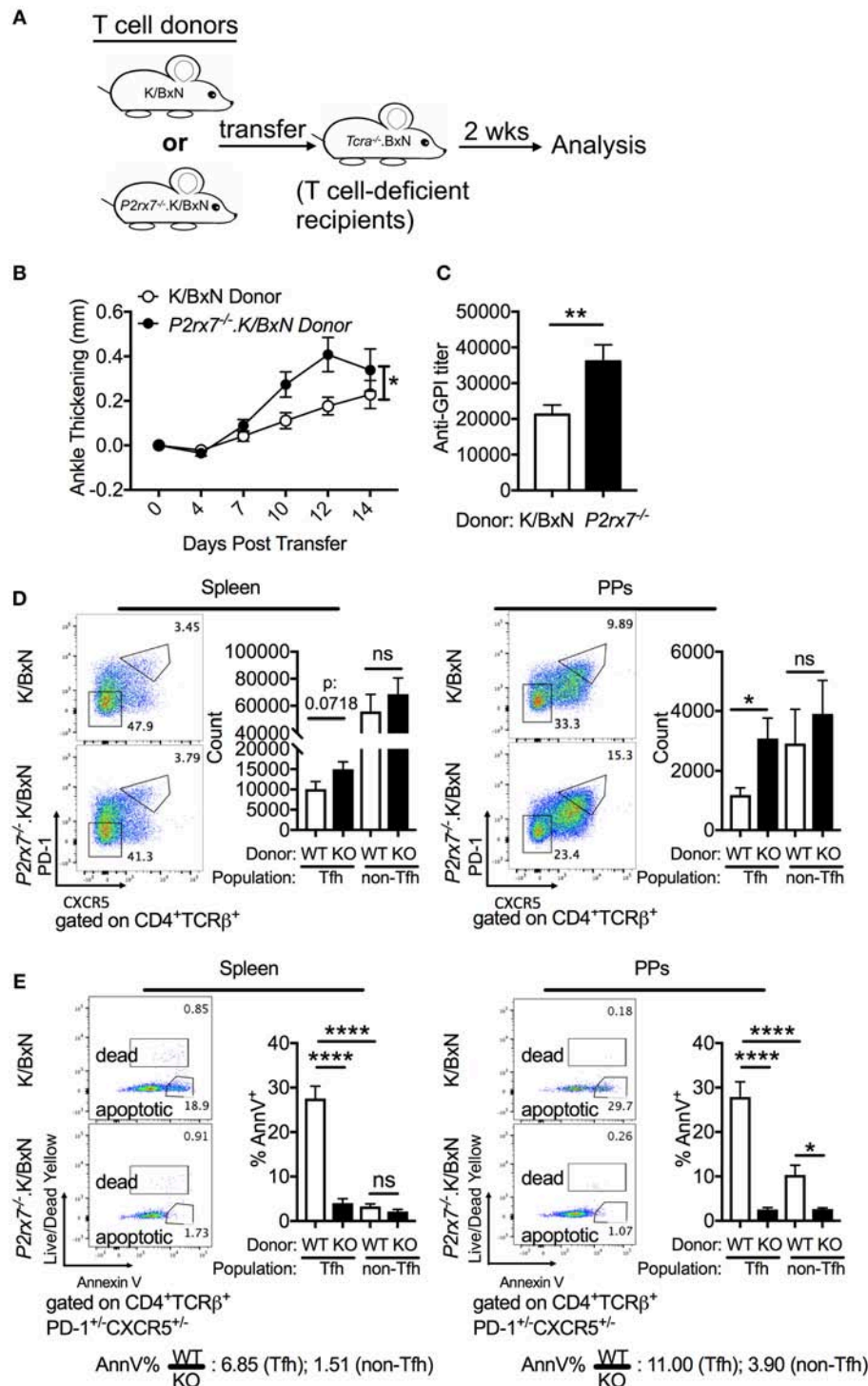
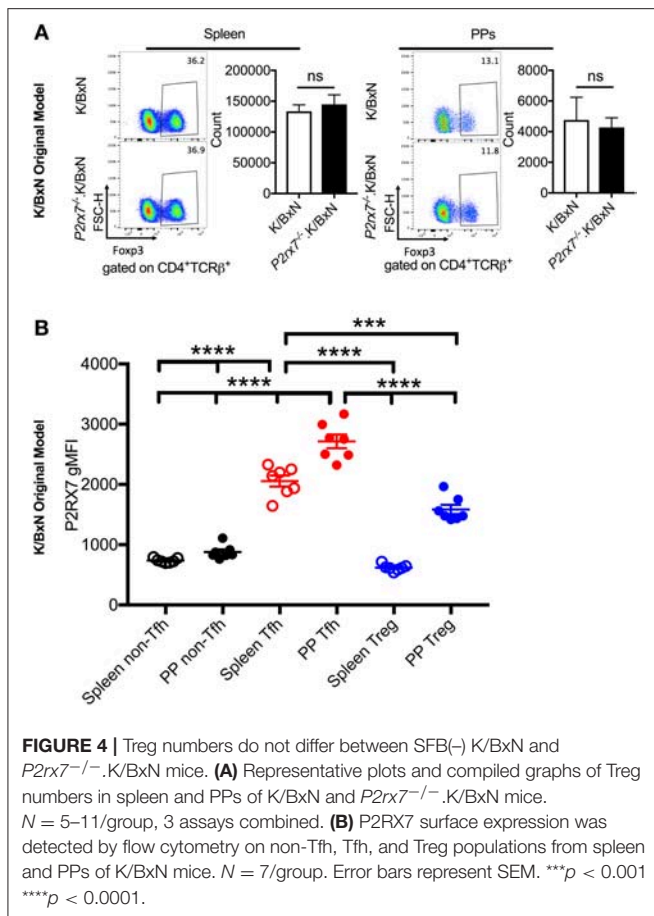


FIGURE 3 | $P2rx7^{-/-}$ T cells enhance autoimmunity in the SFB(-) K/BxN T cell transfer model. **(A–E)** $CD4^{+}$ T cells were isolated from either WT K/BxN or $P2rx7^{-/-}$.K/BxN donors and transferred to $Tcr\alpha^{-/-}$.BxN recipients. **(A)** Schematic for T cell transfer. **(B)** Arthritis development was followed by ankle measurements starting at the day of transfer (day 0). Values are shown as averages of the change in ankle thickness for each individual compared to its first measurement. $N = 20$ –24/group, 5 assays combined. **(C)** Auto-Ab titer as determined by ELISA against GPI. $N = 20$ –24/group, 5 assays combined. **(D)** Representative plots and compiled graphs of Tfh and non-Tfh cell numbers in spleen and PPs. $N = 15$ –18/group, 4 assays combined. **(E)** Representative plots of Tfh and non-Tfh cells stained for Annexin V and Live/Dead Yellow and compiled graphs of Live/Dead Yellow⁺ Annexin V⁺ Tfh or non-Tfh cells as a percentage of total Tfh or non-Tfh cells in spleen and PPs. WT = K/BxN, KO = $P2rx7^{-/-}$.K/BxN. Ratios determined by dividing the average of the WT group by the average of KO group. $N = 8$ –11/group, 3 assays combined. * $p < 0.05$ ** $p < 0.01$ **** $p < 0.0001$.



Also, similar to the whole-mouse knockout, the difference in average percentage of apoptotic splenic Tfh cells between WT and P2RX7-deficient mice (ratio of WT to KO = 6.85) is less compared to that of PP Tfh cells (ratio of WT to KO = 11.00). Interestingly though, the gap between the ratios of spleen and PP in the T cell transfer model (6.85 and 11.0, **Figure 3E**) was less when compared to the whole mouse model (2.53 and 16.37, **Figure 2B**). Additionally, we found that WT non-Tfh cells do not undergo cell death to the same extent as do WT Tfh cells (**Figure 3E**). Furthermore, the difference in apoptosis between WT and *P2rx7*^{-/-} non-Tfh cells was much less (the average ratio of WT to *P2rx7*^{-/-} apoptotic non-Tfh cell frequency was 1.51 in the spleen and 3.90 for PPs) compared to Tfh cells in both spleen and PPs (**Figure 3E**). These results suggested that Tfh cells are more sensitive to P2RX7-mediated apoptosis than non-Tfh cells, with the largest apoptotic difference between WT and *P2rx7*^{-/-} group occurring in PP Tfh cells.

P2RX7 is also associated with increased cell death in T regulatory cells (Tregs) in WT B6 mice (51, 52). To verify that changes in Treg numbers were not responsible for the increased autoimmunity we observed in the *P2rx7*^{-/-}.K/BxN mice, we examined the Treg population in the K/BxN model. We found that there was no significant change in Treg cell numbers in *P2rx7*^{-/-}.K/BxN compared to WT K/BxN mice (**Figure 4A**). We also examined P2RX7 protein levels in splenic and PP Tregs by

flow cytometry with non-Tfh and Tfh cells as control groups. We confirmed that Tfh cells had much higher P2RX7 levels than non-Tfh cells, as in **Figure 2C**. Splenic Tregs expressed low P2RX7 at levels comparable to the non-Tfh cells (**Figure 4B**). Importantly, while PP Tregs expressed P2RX7 at higher levels, it was still lower than P2RX7 expression on splenic Tfh cells, which as a population, display no difference in cell numbers between WT and *P2rx7*^{-/-}.K/BxN mice. This may explain why, analogous to the splenic Tfh cells, there was also no difference in PP Treg numbers between WT and *P2rx7*^{-/-}.K/BxN mice (**Figure 4B**). This is supported by the results of another group, who found that P2RX7 mRNA, while expressed in Tregs, has the highest expression in Tfh cells (26). We additionally found that there was no significant change in Treg numbers in the K/BxN transfer model in mice that received *P2rx7*^{-/-} T cells compared to those that received WT T cells (**Supplementary Figure 3**). Based on these results, we concluded that Treg depletion is not a driving force in the increased autoimmunity in P2RX7 deficiency.

SFB Do Not Further Exacerbate Autoimmune Arthritis in *P2rx7*^{-/-} Mice

Because Tfh cells have been shown to affect the gut microbiota and vice versa (26, 29), we examined whether a disease enhancing gut microbiota might combine with loss of P2RX7 to induce even greater arthritis development. To address this question, we used the gut commensal SFB, which we have previously demonstrated to exacerbate arthritis in the K/BxN system (29, 36). When we colonized K/BxN and *P2rx7*^{-/-}.K/BxN mice with SFB [SFB(+) hereafter], we found that while *P2rx7*^{-/-} mice trended toward faster arthritis development than WT mice, the WT mice rapidly achieved the same level of arthritis (**Figure 5A**). When we analyzed anti-GPI auto-Ab titers at the experiment endpoint (~age 6 weeks old), we found no difference between SFB(+) WT and *P2rx7*^{-/-} mice (**Figure 5B**). We hypothesized that one explanation for the lack of difference between WT and *P2rx7*^{-/-} mice in the SFB(+) condition could be that, as demonstrated by another group, P2RX7 actively regulates IgA levels by controlling the Tfh population, permitting symbiosis with commensal gut microbes, and in *P2rx7*^{-/-} mice, SFB levels decrease due to unregulated IgA production (26). Indeed, when we performed our standard SFB level analysis 10 days after SFB gavage (~age 32 days old) by quantitative PCR in *P2rx7*^{-/-}.K/BxN compared to WT K/BxN mice, we found a decrease in SFB (**Figure 5C**). However, the decreased SFB colonization levels in *P2rx7*^{-/-}.K/BxN mice were still high enough to induce enhanced autoimmunity in our model based on previous results (29).

To further address the interactions of SFB and P2RX7, we used T cell-specific P2RX7 deletion and asked how the gut microbiota might affect the PP Tfh response. *P2rx7*^{-/-} or WT K/BxN T cells were transferred into TCRα^{-/-}.BxN mice pre-colonized with SFB (see **Figure 3A**). Unlike in mice that were not colonized with SFB [SFB(-) hereafter], SFB(+) TCRα^{-/-}.BxN mice that received *P2rx7*^{-/-} T cells displayed no change in arthritis development or auto-Ab titer (**Figures 5D,E** compare **Figures 3B,C**). These data demonstrated, similarly to what we had observed in the complete knockout model, that P2RX7

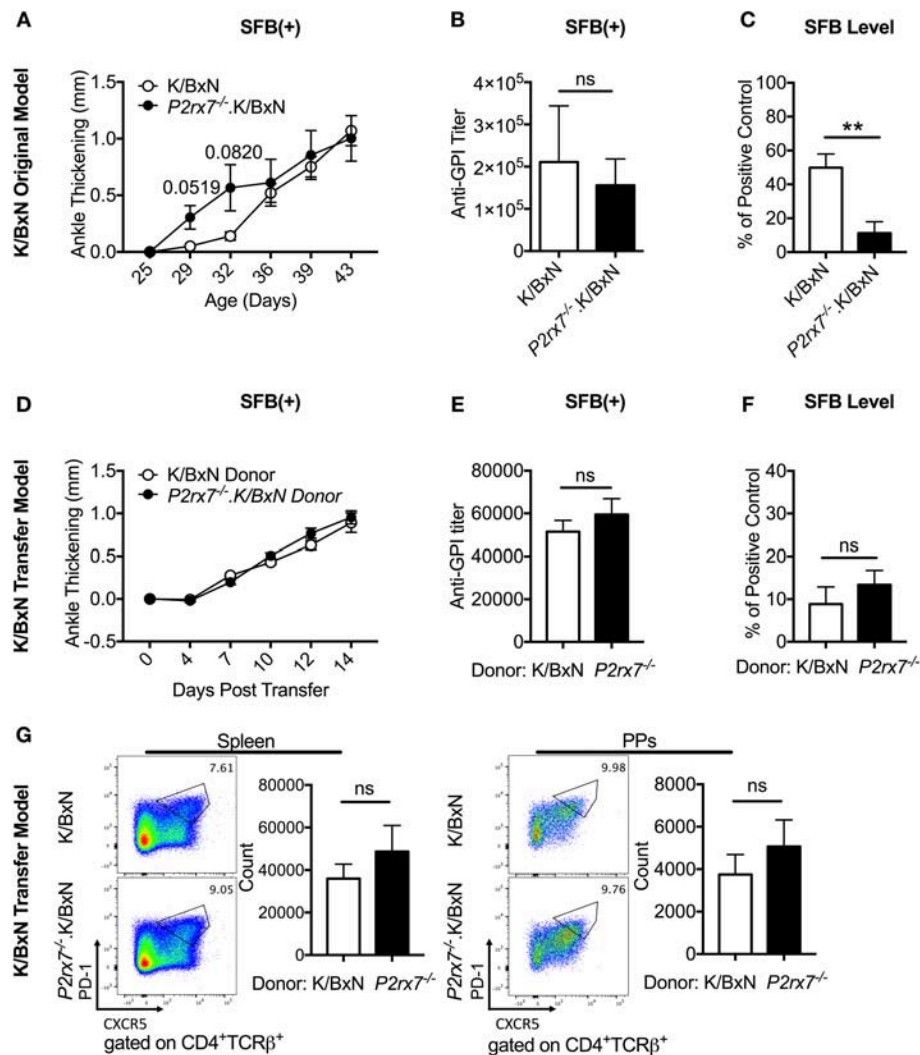


FIGURE 5 | SFB colonization negates the difference in disease severity between WT and *P2rx7*^{-/-} mice in the original K/BxN model and K/BxN T cell transfer model. **(A–G)** K/BxN mice **(A–C)** or TCR α ^{-/-}. BxN mice **(D–G)** were gavaged with SFB 1–2 days after weaning. **(A)** Ankle thickness was followed over time for K/BxN and *P2rx7*^{-/-}.K/BxN mice between ~3.5 and 6 weeks of age. Data are shown as change in ankle thickness compared to first measurement. Error bars represent SEM. *N* = 7–17/group, multiple litters combined. **(B)** Anti-GPI auto-Ab titers in serum obtained from mice 5–7 weeks old were measured by ELISA. *N* = 8–11/group, 5 assays combined. **(C)** SFB level 10 days after gavage was determined by quantitative PCR on 16S rDNA, normalized to a standardized positive control. *N* = 5–12/group, 5 assays combined. **(D–G)** SFB(+) TCR α ^{-/-}.BxN recipients were given either K/BxN or *P2rx7*^{-/-}.K/BxN CD4⁺ cells retro-orbitally at 6 weeks of age. **(D)** Ankle thickness was followed beginning at day of transfer for TCR α ^{-/-}.BxN mice transferred with either K/BxN or *P2rx7*^{-/-}.K/BxN CD4⁺ cells. Data are shown as change in ankle thickness compared to first measurement. Error bars represent SEM. *N* = 12/group, 3 assays combined. **(E)** Anti-GPI auto-Ab titers in serum obtained from the end point of each experiment were measured by ELISA. *N* = 12/group, 3 assays combined. **(F)** SFB level determined by quantitative PCR on 16S rDNA, normalized to a standardized positive control. Feces were collected at end point of each experiment (14 days after T cell transfer which was equivalent to 5 weeks after SFB gavage). *N* = 23–24/group, 5 assays combined. **(G)** Representative plots and compiled graphs of spleen and PP Tfh numbers. *N* = 11/group, 3 assays combined. ***p* < 0.01.

deficiency had no additive effect on disease when combined with SFB colonization. Additionally, we found that unlike in P2RX7 deficiency in the whole organism, in our T cell transfer model, T cell-specific P2RX7 deficiency did not change the SFB level after transfer (Figure 5F). As this study was done in the transfer model, to measure the transferred T cells effects on SFB colonization, we analyzed SFB level at a much later stage (two weeks after transfer which was equivalent to 5 weeks after

SFB gavage) compared to the whole mouse deletion (measured 10 days after SFB gavage, Figure 5C), and thus their SFB levels had declined from initial levels, as has been noted in our previous reports (44). Nevertheless, unlike in whole mouse deletion (Figure 5C), T cell-specific P2RX7 deletion does not further reduce the SFB level (Figure 5F). This suggests that P2RX7 deficiency in T cells alone is not enough to inhibit SFB colonization levels, at least in the K/BxN arthritis model. When

we further examined Tfh cells in SFB(+) transfer mice, we found that although there was a slight upward trend in Tfh numbers in mice that received *P2rx7*^{-/-} T cells compared to those that received WT cells, there was no significant difference in either the spleen or the PPs (Figure 5G).

Up-Regulation of TIGIT Corresponds With a Reduction in Apoptosis in *P2rx7*^{-/-} Tfh Cells

Because we observed enhanced Tfh cell responses and reduced apoptotic Tfh cells in mice with both complete and T cell-intrinsic P2RX7 deficiency under SFB(-) conditions, we next investigated the molecular mechanism whereby P2RX7 exerted its pro-apoptotic effect in SFB(-) mice. In chronic infections and cancer, T cells are exposed to persistent antigens, causing T cell dysfunction, a state called “exhaustion” (53, 54). An important characteristic of exhausted T cells is that these T cells do not undergo cell death, but instead become inactive and unresponsive to stimulation (55). Indeed, activation of TIGIT, a well-known T cell exhaustion marker, up-regulates anti-apoptotic molecules and promotes T cell survival (56). Because of the reduced apoptosis in Tfh cells derived from *P2rx7*^{-/-}.K/BxN mice, we asked whether P2RX7 deficiency in T cells might reduce apoptotic Tfh cells by modulating TIGIT. To investigate this question, we examined the expression levels of TIGIT on *P2rx7*^{-/-} or WT K/BxN T cells transferred into SFB(-) TCRα^{-/-}.BxN mice (57). We found an increase in TIGIT expression on both splenic and PP *P2rx7*^{-/-}.K/BxN Tfh cells compared to WT K/BxN T cells (Figures 6A,B). To further determine the role of TIGIT up-regulation in P2RX7-deficient Tfh cells, we examined cell death between TIGIT⁺ and TIGIT⁻ Tfh cells. We found that splenic and PP TIGIT⁺ Tfh cells, in both mice that received WT and those that received *P2rx7*^{-/-} T cells, were associated with a decrease in apoptosis, as detected by Annexin V staining among Live/Dead Yellow negative cells, compared to TIGIT⁻ Tfh cells (Figures 7A,B). In *P2rx7*^{-/-} TIGIT⁻ Tfh cells, which were already far less susceptible to cell death than WT TIGIT⁻ Tfh cells, the expression of TIGIT was associated with a further reduction in the apoptotic Tfh cell population both in the spleens and in the PPs.

DISCUSSION

P2RX7 has garnered a lot of interest in recent years as a potential therapeutic target for autoimmune arthritis and many other diseases (17, 19, 25). However, the results have been inconsistent and sometimes contradictory. We aimed to further elucidate the role of P2RX7 in autoimmunity by using the spontaneous autoimmune arthritis K/BxN model, along with adoptive transfers to limit the effects of P2RX7 deficiency specifically in T cells. We found that P2RX7 deficiency in the original K/BxN model led to worsened arthritis and higher auto-Ab titers. In addition, examination of Tfh cells revealed the differential impact of P2RX7 in systemic and mucosal tissues: while splenic Tfh cells changed little, PP Tfh cells increased in the absence of P2RX7, despite the fact that Tfh cells in both

organs experienced a disruption of cell death. We further found that T cell-intrinsic P2RX7 ablation is sufficient to generate an autoimmune-exacerbation effect similar to that of P2RX7 deficiency in the whole mouse, i.e., an increase in arthritis, auto-Ab titers, and PP Tfh cell numbers. Many P2RX7 antagonists were designed for anti-inflammatory purposes (19). Our finding that P2RX7 deficiency mediated autoimmune arthritis counters these anti-inflammatory purposes, as we demonstrated that P2RX7 deficiency enhances rather than reduces autoimmune arthritis. We believe the conflicting beneficial or detrimental effects of P2RX7 inhibition could be based on the various etiopathogenesis in different diseases. For example, P2RX7 inhibition would likely enhance those autoimmune diseases that are mediated by auto-Abs and Tfh cells such as is seen here in the K/BxN model and in EAE models reported previously (21). In contrast, diseases that are mediated by innate immune players may be ameliorated by P2RX7 inhibition, which targets the IL-1 and IL-18 pathways (19).

Interestingly, when we colonized mice with SFB in either our K/BxN or T cell transfer model, we found that SFB colonization negated the difference observed between WT and P2RX7-deficient mice. We have previously reported that SFB enhanced the PP Tfh cell response in WT K/BxN mice and the K/BxN T cell transfer model, and PP Tfh cells are required for SFB-mediated arthritis enhancement (29). SFB-mediated disease enhancement can also be demonstrated when compared arthritis and anti-GPI titers between SFB (-) and (+) WT K/BxN mice in the current study (Figures 1A,B vs. Figures 5A,B). Thus, we suspect the lack of disease difference between SFB (+) WT and P2RX7-deficient mice is most likely because SFB do not further increase the PP Tfh cell response in P2RX7 deficient mice (compare Figure 3D and Figure 5G). We speculate that the lack of arthritis difference between WT and KO groups in the SFB (+) condition could be due to an overlapping effect between SFB and P2RX7 deficiency in enhancing arthritis development. More studies will be required to determine the exact mechanism of whether and how SFB may inhibit P2RX7 signaling to enhance Tfh cell responses and arthritis development. Our data also suggested that gut microbiota composition may account for some of the disparities in P2RX7 effects in previous studies. Notably, microbe-host interactions where the host impacts the microbiota have also been observed in regard to P2RX7 deficiency. For example, P2RX7 deficiency has been previously demonstrated to trigger an enhanced PP Tfh cell response, leading to greater IgA production by B cells to inhibit SFB colonization (26). This effect likely requires the combination of T cell-specific and B cell-specific P2RX7 deficiency, as we found that SFB colonization was reduced with whole-mouse but not T cell-intrinsic P2RX7 deficiency. Our T cell transfer data showing no difference in arthritis (Figure 5D) and SFB level (Figure 5F) between P2RX7-deficient and WT groups indicate that lower SFB levels in whole-mouse P2RX7 deficiency (Figure 5C) was not solely responsible for the lack of arthritis difference between WT and *P2rx7*^{-/-} mice in SFB(+) condition (Figures 5A,D) when compared to SFB(-) condition (Figures 1A, 3B). Future studies will be required to understand the mechanisms involved in microbiota-mediated autoimmunity in the absence of P2RX7.

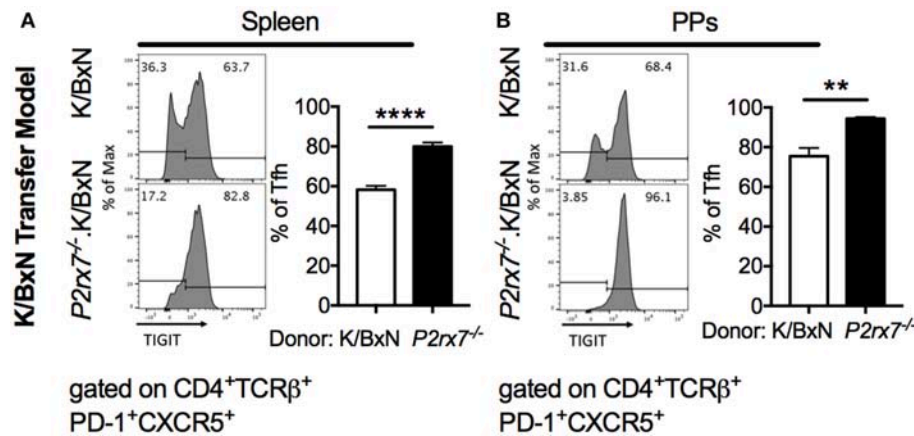


FIGURE 6 | TIGIT is increased on $P2rx7^{-/-}$ Tfh cells in the SFB(-) K/BxN transfer model. **(A,B)** Representative histograms and compiled graphs of TIGIT expression on **(A)** splenic or **(B)** PP Tfh cells from WT or $P2rx7^{-/-}$.K/BxN $CD4^{+}$ cells transferred into $TCR\alpha^{-/-}$.BxN mice. Compiled graphs show TIGIT $^{+}$ Tfh as a percentage of total Tfh cells. $N = 7-9/\text{group}$, 2 assays combined. $^{*}p < 0.01$ $^{****}p < 0.0001$.

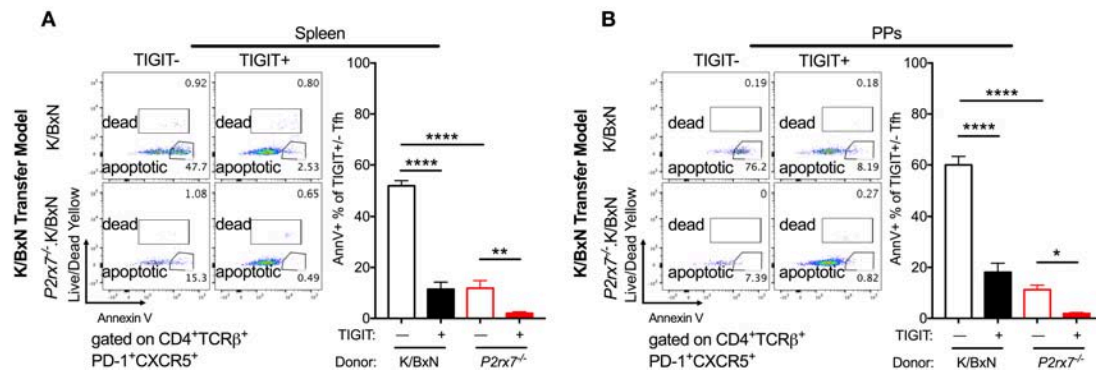
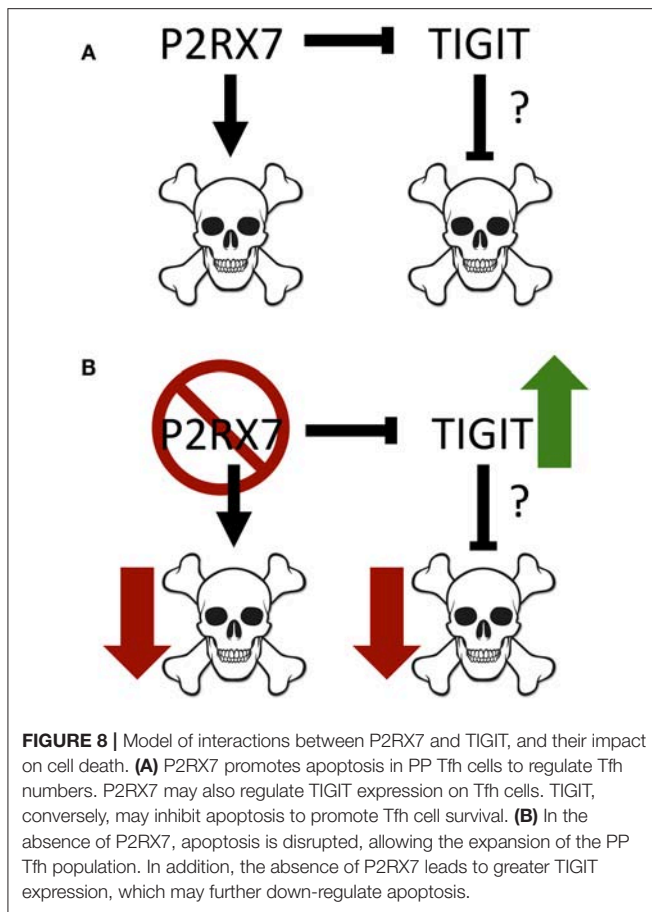


FIGURE 7 | TIGIT expression on Tfh cells is associated with decreased apoptosis in the SFB(-) K/BxN transfer model. **(A,B)** WT or $P2rx7^{-/-}$.K/BxN $CD4^{+}$ cells were transferred into $TCR\alpha^{-/-}$. BxN recipients. Shown are representative plots and compiled graphs of Live/Dead Yellow $^{-}$ Annexin V $^{+}$ staining as a percentage of either TIGIT $^{+}$ or TIGIT $^{-}$ Tfh cells. $N = 11-14/\text{group}$, 3 assays combined. $^{*}p < 0.05$ $^{**}p < 0.01$ $^{****}p < 0.0001$.

Notably, we identified a significant increase in TIGIT expression on $P2rx7^{-/-}$ Tfh cells. As mentioned earlier, activation of TIGIT up-regulates anti-apoptotic molecules and promotes T cell survival (56). In $P2rx7^{-/-}$ TIGIT $^{-}$ Tfh cells, which were already far less susceptible to cell death than WT TIGIT $^{-}$ Tfh cells, the expression of TIGIT was associated with a further reduction of the apoptotic Tfh cell population both in the spleens and in the PPs (**Figure 8**). These data suggested that P2RX7 can promote cell death in the Tfh population through a TIGIT-independent mechanism (**Figure 8**). Our data further suggested that P2RX7 could potentially promote cell death in the Tfh population by down-regulating TIGIT and inhibiting TIGIT's anti-apoptotic effect. Clearly, more future studies need to be done to address the apoptotic regulation by P2RX7 and TIGIT. TIGIT has historically been viewed as an inhibitory molecule, which signals via ITIM domains to inhibit T cell activation (56, 57). However, more recent work has identified TIGIT $^{+}$ Tfh cells as drivers of the immune response (58, 59). TIGIT, though

it has been associated with T cell exhaustion, may have other roles as well. A pathogenic role for TIGIT has been implicated by two other groups, one of which demonstrated that a pathogenic subset of Tfh cells circulating in human RA patients' blood can be identified in part by enhanced expression of TIGIT (59), and the other of which demonstrated increased B cell costimulatory activity in a TIGIT $^{+}$ subset of Tfh cells, which is inhibited by anti-TIGIT blocking antibodies (58).

Consistent with previous results from another group (26), we found an induction of the PP Tfh population and little change in the splenic Tfh response in $P2rx7^{-/-}$.K/BxN mice compared to K/BxN mice. One explanation for the difference between the mucosal and systemic sites could be the much higher P2RX7 expression in PP compared to splenic Tfh cells (>2-fold), as detected in the whole-mouse model. This would make PP Tfh cells more sensitive to P2RX7-induced cell death than splenic Tfh cells, as the ratio of WT to $P2rx7^{-/-}$.K/BxN apoptotic Tfh cells in the spleen compared to the PPs is 2.53 and 16.37, respectively,



indicating greater P2RX7-mediated apoptosis in the PPs than the spleen. We have further confirmed the PP Tfh induction phenotype using *P2rx7*^{-/-}.K/BxN T cells in the transfer model. Interestingly, T cell-intrinsic P2RX7 deficiency induced the PP Tfh response and it almost significantly induced the Tfh cell response in the spleen as well. This corresponded with the increased ratio of WT to *P2rx7*^{-/-}.K/BxN apoptotic Tfh cells in spleen of T cell-specific deletion experiments when compared to that of whole mouse deletion experiments (2.53). These results suggested that P2RX7 deficiency in T cells alone allow T cells to be more sensitive to P2RX7-mediated deletion in systemic sites. Finally, regardless of the system, there were higher ratios of WT to *P2rx7*^{-/-}.K/BxN apoptotic Tfh cells in PPs compared to the spleen. This may be due to the unique environment of the PPs, because commensal bacteria can serve as a major source of intestinal luminal ATP to activate P2RX7 (60, 61).

Similarly to Tfh cells, P2RX7 has generally been associated with increased cell death in Tregs (51, 52). Thus, the effect

of P2RX7 deficiency on Tregs would likely increase Treg cell numbers, and would be unlikely to explain the increase in autoimmunity observed in *P2rx7*^{-/-}.K/BxN mice. In the K/BxN autoimmune model, we did not observe a difference in Treg cell number between WT and *P2rx7*^{-/-} groups. Thus, it appears that in WT B6 mice, Tregs are more sensitive to P2RX7-induced apoptosis than the Tregs under autoimmune conditions. P2RX7 inhibits mTOR, which is a critical regulator of Tfh cells (62, 63). Activation of mTORC1 and mTORC2 expands Tfh and depletes regulatory T cells (64). Due to limitations on the scope of this study, we did not investigate the role of mTOR in P2RX7-mediated apoptosis. Undoubtedly, more studies will be required to address this important question.

In conclusion, contrary to the expected anti-inflammatory effect of P2RX7 inhibition through down-regulating innate immune responses (19), T cell-specific P2RX7 inhibition effectively enhanced K/BxN inflammatory arthritis. This effect was associated with an enhancement of the mucosal (but not systemic) Tfh cell response. These data suggest that non-selective P2RX7 antagonists may not be very effective in ameliorating autoimmune diseases, which may explain the failures in previous P2RX7-antagonist clinical trials with RA. Interestingly, a recent report suggests that P2RX7 is required for the establishment, maintenance and functionality of long-lived memory CD8⁺ T cell populations but not short-lived effector CD8⁺ T cells (65). These findings suggest that activation of P2RX7 by extracellular ATP can generate a variety of, or even opposite, outcomes based on the cell type (66). Our results illustrate a valuable lesson that cell-specific targeting of P2RX7 should be considered in order to achieve efficacy for P2RX7-related therapy.

AUTHOR CONTRIBUTIONS

KE, FT, and H-JW conceived and designed the study and wrote the manuscript. KE, FT, and NB designed and performed the experiments and analyzed the data. HM, IJ, KS, and NT performed SFB quantification, genotyping and ELISA analysis.

FUNDING

This work was supported by funds from NIEHS (R21ES027547) and NIAID (R01AI107117) to H-JW.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2019.00411/full#supplementary-material>

REFERENCES

1. Silman AJ, Macgregor AJ, Thomson W, Holligan S, Carthy D, Farhan A, et al. Twin concordance rates for rheumatoid arthritis: results from a nationwide study. *Br J Rheumatol.* (1993) 32:903–7. doi: 10.1093/rheumatology/32.10.903

2. Seldin ME, Amos CI, Ward R, Gregersen PK. The genetics revolution and the assault on rheumatoid arthritis. *Arthritis Rheum.* (1999) 42:1071–9. doi: 10.1002/1529-0131(199906)42:6<1071::AID-ANR1>3.0.CO;2-8
3. Wu HJ, Wu E. The role of gut microbiota in immune homeostasis and autoimmunity. *Gut Microbes.* (2012) 3:4–14. doi: 10.4161/gmic.19320

4. Cross M, Smith E, Hoy D, Carmona L, Wolfe F, Vos T, et al. The global burden of rheumatoid arthritis: estimates from the global burden of disease 2010 study. *Ann Rheum Dis.* (2014) 73:1316–22. doi: 10.1136/annrheumdis-2013-204627
5. Smolen JS, Aletaha D, McInnes IB. Rheumatoid arthritis. *Lancet.* (2016) 388:2023–38. doi: 10.1016/S0140-6736(16)30173-8
6. Felix KM, Tahsin S, Wu HJ. Host-microbiota interplay in mediating immune disorders. *Ann N Y Acad Sci.* (2018) 1417:57–70. doi: 10.1111/nyas.13508
7. Pekin TJ Jr, Zvaifler NJ. Hemolytic complement in synovial fluid. *J Clin Invest.* (1964) 43:1372–82. doi: 10.1172/JCI105013
8. Ruddy S, Austen KF. The complement system in rheumatoid synovitis. I An analysis of complement component activities in rheumatoid synovial fluids. *Arthritis Rheum.* (1970) 13:713–23. doi: 10.1002/art.1780130601
9. Britton MC, Schur PH. The complement system in rheumatoid synovitis. II intracytoplasmic inclusions of immunoglobulins and complement. *Arthritis Rheum.* (1971) 14:87–95. doi: 10.1002/art.1780140111
10. Halla JT, Volanakis JE, Schrohenloher RE. Immune complexes in rheumatoid arthritis sera and synovial fluids: a comparison of three methods. *Arthritis Rheum.* (1979) 22:440–8. doi: 10.1002/art.1780220502
11. Olsen NJ, Ho E, Barats L. Clinical correlations with serum C1q levels in patients with rheumatoid arthritis. *Arthritis Rheum.* (1991) 34:187–91. doi: 10.1002/art.1780340209
12. Mathsson L, Lampa J, Mullazehi M, Ronnelid J. Immune complexes from rheumatoid arthritis synovial fluid induce FcγRIIIa dependent and rheumatoid factor correlated production of tumour necrosis factor-α by peripheral blood mononuclear cells. *Arthritis Res Ther.* (2006) 8:R64. doi: 10.1186/ar1926
13. Crotty S. Follicular helper CD4 T cells (TFH). *Annu Rev Immunol.* (2011) 29:621–63. doi: 10.1146/annurev-immunol-031210-101400
14. Ma CS, Deenick EK, Batten M, Tangye SG. The origins, function, and regulation of T follicular helper cells. *J Exp Med.* (2012) 209:1241–53. doi: 10.1084/jem.20120994
15. Crotty S. T follicular helper cell differentiation, function, and roles in disease. *Immunity.* (2014) 41:529–42. doi: 10.1016/j.immuni.2014.10.004
16. Ueno H, Banchereau J, Vinuesa CG. Pathophysiology of T follicular helper cells in humans and mice. *Nat Immunol.* (2015) 16:142–52. doi: 10.1038/ni.3054
17. Di Virgilio F, Dal Ben D, Sarti AC, Giuliani AL, Falzoni S. The P2X7 receptor in infection and inflammation. *Immunity.* (2017) 47:15–31. doi: 10.1016/j.immuni.2017.06.020
18. Ghiringhelli F, Apetoh L, Tesniere A, Aymeric L, Ma Y, Ortiz C, et al. Activation of the NLRP3 inflammasome in dendritic cells induces IL-1β-dependent adaptive immunity against tumors. *Nat Med.* (2009) 15:1170–8. doi: 10.1038/nm.2028
19. Bartlett R, Stokes L, Sluyter R. The P2X7 receptor channel: recent developments and the use of P2X7 antagonists in models of disease. *Pharmacol Rev.* (2014) 66:638–75. doi: 10.1124/pr.113.008003
20. Labasi JM, Petrushova N, Donovan C, McCurdy S, Lira P, Payette MM, et al. Absence of the P2X7 receptor alters leukocyte function and attenuates an inflammatory response. *J Immunol.* (2002) 168:6436–45. doi: 10.4049/jimmunol.168.12.6436
21. Chen L, Brosnan CF. Exacerbation of experimental autoimmune encephalomyelitis in P2X7R^{-/-} mice: evidence for loss of apoptotic activity in lymphocytes. *J Immunol.* (2006) 176:3115–26. doi: 10.4049/jimmunol.176.5.3115
22. Chen YG, Scheuplein F, Driver JP, Hewes AA, Reifsnnyder PC, Leiter EH, et al. Testing the role of P2X7 receptors in the development of type 1 diabetes in nonobese diabetic mice. *J Immunol.* (2011) 186:4278–84. doi: 10.4049/jimmunol.1003733
23. Keystone EC, Wang MM, Layton M, Hollis S, McInnes IB, Team DCS. Clinical evaluation of the efficacy of the P2X7 purinergic receptor antagonist AZD9056 on the signs and symptoms of rheumatoid arthritis in patients with active disease despite treatment with methotrexate or sulphasalazine. *Ann Rheum Dis.* (2012) 71:1630–5. doi: 10.1136/annrheumdis-2011-143578
24. Stock TC, Bloom BJ, Wei N, Ishaq S, Park W, Wang X, et al. Efficacy and safety of CE-224,535, an antagonist of P2X7 receptor, in treatment of patients with rheumatoid arthritis inadequately controlled by methotrexate. *J Rheumatol.* (2012) 39:720–7. doi: 10.3899/jrheum.110874
25. Cekic C, Linden J. Purinergic regulation of the immune system. *Nat Rev Immunol.* (2016) 16:177–92. doi: 10.1038/nri.2016.4
26. Proietti M, Cornacchione V, Rezzonico Jost T, Romagnani A, Faliti CE, Perruzza L, et al. ATP-gated ionotropic P2X7 receptor controls follicular T helper cell numbers in Peyer's patches to promote host-microbiota mutualism. *Immunity.* (2014) 41:789–801. doi: 10.1016/j.immuni.2014.10.010
27. Salles EM, Menezes MN, Siqueira R, Borges Da Silva H, Amaral EP, Castillo-Mendez SI, et al. P2X7 receptor drives Th1 cell differentiation and controls the follicular helper T cell population to protect against *Plasmodium chabaudi* malaria. *PLoS Pathog.* (2017) 13:e1006595. doi: 10.1371/journal.ppat.1006595
28. Gattorno M, Piccini A, Lasiglie D, Tassi S, Brisca G, Carta S, et al. The pattern of response to anti-interleukin-1 treatment distinguishes two subsets of patients with systemic-onset juvenile idiopathic arthritis. *Arthritis Rheum.* (2008) 58:1505–15. doi: 10.1002/art.23437
29. Teng F, Klinger CN, Felix KM, Bradley CP, Wu E, Tran NL, et al. Gut microbiota drive autoimmune arthritis by promoting differentiation and migration of Peyer's patch T follicular helper cells. *Immunity.* (2016) 44:875–88. doi: 10.1016/j.immuni.2016.03.013
30. Monach PA, Mathis D, Benoist C. The K/BxN arthritis model. *Curr Protoc Immunol Chapter.* (2008) 15:1522. doi: 10.1002/0471142735.im1522s81
31. Ditzel HJ. The K/BxN mouse: a model of human inflammatory arthritis. *Trends Mol Med.* (2004) 10:40–5. doi: 10.1016/j.molmed.2003.11.004
32. Kouskoff V, Korganow AS, Duchatelle V, Degott C, Benoist C, Mathis D. Organ-specific disease provoked by systemic autoimmunity. *Cell.* (1996) 87:811–22. doi: 10.1016/S0092-8674(00)81989-3
33. Korganow AS, Ji H, Mangialaio S, Duchatelle V, Pelanda R, Martin T, et al. From systemic T cell self-reactivity to organ-specific autoimmune disease via immunoglobulins. *Immunity.* (1999) 10:451–61. doi: 10.1016/S1074-7613(00)80045-X
34. Matsumoto I, Staub A, Benoist C, Mathis D. Arthritis provoked by linked T and B cell recognition of a glycolytic enzyme. *Science.* (1999) 286:1732–5. doi: 10.1126/science.286.5445.1732
35. Bradley CP, Teng F, Felix KM, Sano T, Naskar D, Block KE, et al. Segmented filamentous bacteria provoke lung autoimmunity by inducing gut-lung axis Th17 cells expressing dual TCRs. *Cell Host Microbe.* (2017) 22:697–704 e694. doi: 10.1016/j.chom.2017.10.007
36. Wu HJ, Ivanov I, Darce J, Hattori K, Shima T, Umesaki Y, et al. Gut-residing segmented filamentous bacteria drive autoimmune arthritis via T helper 17 cells. *Immunity.* (2010) 32:815–27. doi: 10.1016/j.immuni.2010.06.001
37. Adriouch S, Dox C, Welge V, Seman M, Koch-Nolte F, Haag F. Cutting edge: a natural P451L mutation in the cytoplasmic domain impairs the function of the mouse P2X7 receptor. *J Immunol.* (2002) 169:4108–12. doi: 10.4049/jimmunol.169.8.4108
38. Le Stunff H, Auger R, Kanellopoulos J, Raymond MN. The Pro-451 to Leu polymorphism within the C-terminal tail of P2X7 receptor impairs cell death but not phospholipase D activation in murine thymocytes. *J Biol Chem.* (2004) 279:16918–26. doi: 10.1074/jbc.M313064200
39. Young MT, Pelegrin P, Surprenant A. Identification of Thr283 as a key determinant of P2X7 receptor function. *Br J Pharmacol.* (2006) 149:261–8. doi: 10.1038/sj.bjp.0706880
40. Donnelly-Roberts DL, Namovic MT, Han P, Jarvis MF. Mammalian P2X7 receptor pharmacology: comparison of recombinant mouse, rat and human P2X7 receptors. *Br J Pharmacol.* (2009) 157:1203–14. doi: 10.1111/j.1476-5381.2009.00233.x
41. Dan JM, Lindestam Arlehamn CS, Weiskopf D, Da Silva Antunes R, Haverndar-Daughton C, Reiss SM, et al. A cytokine-independent approach to identify antigen-specific human germinal center T follicular helper cells and rare antigen-specific CD4⁺ T Cells in blood. *J Immunol.* (2016) 197:983–93. doi: 10.4049/jimmunol.1600318
42. Haverndar-Daughton C, Lindqvist M, Heit A, Wu JE, Reiss SM, Kendrick K, et al. CXCL13 is a plasma biomarker of germinal center activity. *Proc Natl Acad Sci USA.* (2016) 113:2702–7. doi: 10.1073/pnas.1520112113
43. Naskar D, Teng F, Felix KM, Bradley CP, Wu HJ. Synthetic retinoid AM80 ameliorates lung and arthritic autoimmune responses by inhibiting T follicular helper and Th17 cell responses. *J Immunol.* (2017) 198:1855–64. doi: 10.4049/jimmunol.1601776
44. Teng F, Felix KM, Bradley CP, Naskar D, Ma H, Raslan WA, et al. The impact of age and gut microbiota on Th17 and Tfh cells in K/BxN

- autoimmune arthritis. *Arthritis Res Ther.* (2017) 19:188. doi: 10.1186/s13075-017-1398-6
45. Gaddis DE, Padgett LE, Wu R, McSkimming C, Romines V, Taylor AM, et al. Apolipoprotein AI prevents regulatory to follicular helper T cell switching during atherosclerosis. *Nat Commun.* (2018) 9:1095. doi: 10.1038/s41467-018-03493-5
 46. Wang Y, Gao W, Shi X, Ding J, Liu W, He H, et al. Chemotherapy drugs induce pyroptosis through caspase-3 cleavage of a gasdermin. *Nature.* (2017) 547:99–103. doi: 10.1038/nature22393
 47. Humphreys BD, Dubyak GR. Modulation of P2X7 nucleotide receptor expression by pro- and anti-inflammatory stimuli in THP-1 monocytes. *J Leukoc Biol.* (1998) 64:265–73. doi: 10.1002/jlb.64.2.265
 48. Browning JL. B cells move to centre stage: novel opportunities for autoimmune disease treatment. *Nat Rev Drug Discov.* (2006) 5:564–76. doi: 10.1038/nrd2085
 49. Nurieva RI, Chung Y, Martinez GJ, Yang XO, Tanaka S, Matskevitch TD, et al. Bcl6 mediates the development of T follicular helper cells. *Science.* (2009) 325:1001–5. doi: 10.1126/science.1176676
 50. Labranche TP, Hickman-Brecks CL, Meyer DM, Storer CE, Jesson MI, Shevlin KM, et al. Characterization of the KRN cell transfer model of rheumatoid arthritis (KRN-CTM), a chronic yet synchronized version of the K/BxN mouse. *Am J Pathol.* (2010) 177:1388–96. doi: 10.2353/ajpath.2010.100195
 51. Aswad F, Kawamura H, Dennert G. High sensitivity of CD4+CD25+ regulatory T cells to extracellular metabolites nicotinamide adenine dinucleotide and ATP: a role for P2X7 receptors. *J Immunol.* (2005) 175:3075–83. doi: 10.4049/jimmunol.175.5.3075
 52. Schenk U, Frascoli M, Proietti M, Geffers R, Traggiai E, Buer J, et al. ATP inhibits the generation and function of regulatory T cells through the activation of purinergic P2X receptors. *Sci Signal.* (2011) 4:ra12. doi: 10.1126/scisignal.2001270
 53. Pauken KE, Wherry EJ. Overcoming T cell exhaustion in infection and cancer. *Trends Immunol.* (2015) 36:265–76. doi: 10.1016/j.it.2015.02.008
 54. Wherry EJ, Kurachi M. Molecular and cellular insights into T cell exhaustion. *Nat Rev Immunol.* (2015) 15:486–99. doi: 10.1038/nri3862
 55. McKinney EF, Smith KGC. Metabolic exhaustion in infection, cancer and autoimmunity. *Nat Immunol.* (2018) 19:213–21. doi: 10.1038/s41590-018-0045-y
 56. Joller N, Hafler JP, Bryneda B, Kassam N, Spoerl S, Levin SD, et al. Cutting edge: TIGIT has T cell-intrinsic inhibitory functions. *J Immunol.* (2011) 186:1338–42. doi: 10.4049/jimmunol.1003081
 57. Anderson AC, Joller N, Kuchroo VK. Lag-3, Tim-3, and TIGIT: co-inhibitory receptors with specialized functions in immune regulation. *Immunity.* (2016) 44:989–1004. doi: 10.1016/j.immuni.2016.05.001
 58. Godefroy E, Zhong H, Pham P, Friedman D, Yazdanbakhsh K. TIGIT-positive circulating follicular helper T cells display robust B-cell help functions: potential role in sickle cell alloimmunization. *Haematologica.* (2015) 100:1415–25. doi: 10.3324/haematol.2015.132738
 59. Rao DA, Gurish MF, Marshall JL, Slowikowski K, Fonseka CY, Liu Y, et al. Pathologically expanded peripheral T helper cell subset drives B cells in rheumatoid arthritis. *Nature.* (2017) 542:110–4. doi: 10.1038/nature20810
 60. Atarashi K, Nishimura J, Shima T, Umesaki Y, Yamamoto M, Onoue M, et al. ATP drives lamina propria T(H)17 cell differentiation. *Nature.* (2008) 455:808–12. doi: 10.1038/nature07240
 61. Lalles JP. Luminal ATP: the missing link between intestinal alkaline phosphatase, the gut microbiota, and inflammation? *Am J Physiol Gastrointest Liver Physiol.* (2014) 306:G824–5. doi: 10.1152/ajpgi.00435.2013
 62. Kim JE, Ko AR, Hyun HW, Min SJ, Kang TC. P2RX7-MAPK1/2-SP1 axis inhibits MTOR independent HSPB1-mediated astroglial autophagy. *Cell Death Dis.* (2018) 9:546. doi: 10.1038/s41419-018-0586-x
 63. Zeng H, Cohen S, Guy C, Shrestha S, Neale G, Brown SA, et al. mTORC1 and mTORC2 kinase signaling and glucose metabolism drive follicular helper T cell differentiation. *Immunity.* (2016) 45:540–54. doi: 10.1016/j.immuni.2016.08.017
 64. Kato H, Perl A. Blockade of treg cell differentiation and function by the interleukin-21-mechanistic target of rapamycin axis via suppression of autophagy in patients with systemic lupus erythematosus. *Arthritis Rheum.* (2018) 70:427–38. doi: 10.1002/art.40380
 65. Borges Da Silva H, Beura LK, Wang H, Hanse EA, Gore R, Scott MC, et al. The purinergic receptor P2RX7 directs metabolic fitness of long-lived memory CD8(+) T cells. *Nature.* (2018) 559:264–8. doi: 10.1038/s41586-018-0282-0
 66. Savio LEB, de Andrade MP, da Silva CG, Coutinho-Silva R. The P2X7 receptor in inflammatory diseases: angel or demon? *Front Pharmacol.* (2018) 9:52. doi: 10.3389/fphar.2018.00052

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2019 Felix, Teng, Bates, Ma, Jaimez, Sleiman, Tran and Wu. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



The Transcription Factor T-Bet Is Required for Optimal Type I Follicular Helper T Cell Maintenance During Acute Viral Infection

Pengcheng Wang^{1,2†}, Youping Wang^{1†}, Luoyingzi Xie^{1†}, Minglu Xiao¹, Jialin Wu¹, Lifan Xu¹, Qiang Bai¹, Yaxing Hao¹, Qizhao Huang³, Xiangyu Chen¹, Ran He¹, Baohua Li¹, Sen Yang⁴, Yaokai Chen^{4*}, Yuzhang Wu^{1*} and Lilin Ye^{1*}

¹ Institute of Immunology, PLA, Third Military Medical University, Chongqing, China, ² National Clinical Research Center of Kidney Diseases, Jinling Hospital, Nanjing, China, ³ Cancer Center, The General Hospital of Western Theater Command, Chengdu, China, ⁴ Chongqing Public Health Medical Center, Chongqing, China

OPEN ACCESS

Edited by:

Georgia Fousteri,
San Raffaele Hospital (IRCCS), Italy

Reviewed by:

Masato Kubo,
Tokyo University of Science, Japan
Colleen Jean Winstead,
Merck Sharp & Dohme Corp,
United States

*Correspondence:

Yaokai Chen
yaokaichen@hotmail.com
Yuzhang Wu
wuyuzhang@tmmu.edu.cn
Lilin Ye
yellilincmv@tmmu.edu.cn

[†]These authors have contributed
equally to this work

Specialty section:

This article was submitted to
T Cell Biology,
a section of the journal
Frontiers in Immunology

Received: 28 November 2018

Accepted: 07 March 2019

Published: 29 March 2019

Citation:

Wang P, Wang Y, Xie L, Xiao M, Wu J, Xu L, Bai Q, Hao Y, Huang Q, Chen X, He R, Li B, Yang S, Chen Y, Wu Y and Ye L (2019) The Transcription Factor T-Bet Is Required for Optimal Type I Follicular Helper T Cell Maintenance During Acute Viral Infection. *Front. Immunol.* 10:606. doi: 10.3389/fimmu.2019.00606

Follicular helper T cells (TFH cells), known as the primary “helpers” of the germinal center (GC) reaction, promote the humoral immune response to defend against various pathogens. Under conditions of infection by different types of pathogens, many shared transcription factors (TFs), such as Bcl-6, TCF-1, and Maf, are selectively enriched in pathogen-specific TFH cells, orchestrating TFH cell differentiation and function. In addition, TFH cells also coexpress environmentally associated TFs as their conventional T cell counterparts (such as T-bet, GATA-3, or ROR- γ t, which are expressed in Th1, Th2, or Th17 cells, respectively). These features likely indicate both the lineage-specificity and environmental adaption of the TFH cell responses. However, the extent to which the TFH cell response relies on these environmentally specific TFs is not completely understood. Here, we found that T-bet was specifically expressed in Type I TFH cells but not Type II TFH cells. While dispensable for the early fate commitment of TFH cells, T-bet was essential for the maintenance of differentiated TFH cells, promoting their proliferation, and inhibiting their apoptosis during acute viral infection. Microarray analysis showed both similarities and differences in transcriptome dependency on T-bet in TFH and TH1 cells, suggesting the distinctive role of T-bet in TFH cells. Collectively, our findings reveal an important and specific supporting role for T-bet in type I TFH cell response, which can help us gain a deeper understanding of TFH cell subsets.

Keywords: T-bet, follicular helper T cells, type I immune response, humoral response, T cell differentiation, transcriptional regulation

INTRODUCTION

Because of the complexity and diversity of pathogens, organisms have developed highly organized and well-adapted immune systems to eliminate invaders. To defend against different microorganisms, the immune system elicits optimal responses according to the species of invader (1). For example, intracellular microbes induce type I immune response, which consists of IFN- γ -producing group1 innate lymphoid cell (ILC) lineages (including natural killer cells and ILC1s) (2–4), CD4⁺ type I T helper cells (TH1) (5, 6), and CD8⁺ type I cytotoxic T cells (TC1) mediated

responses (7, 8); venoms or helminthes induce type II immune response, which includes IL-4-producing ILC2s (9–11), TH2 cells (6, 12), and TC2 cells (7, 13); and extracellular fungi or bacteria induce type III immune response, which comprises IL-17-producing ILC3s (14, 15), TH17 cells (16–18), and TC17 cells (19, 20). This phenomenon reflects the high plasticity and environmental dependency of immune cells.

As a CD4⁺ helper T cell subset specialized to “help” the germinal center (GC) reaction (21, 22), follicular helper T cells (TFH cells) have been reported to play important roles in type I immune response (23–27), type II immune response (28–32), and type III immune response (33–35). TFH cells express high levels of CXCR5, which is required for their localization in lymphoid follicles (21, 22, 36–40). In the light zone of GCs, they provide crucial signals to antigen-specific B cells and promote somatic hyper mutation, class switch recombination (CSR), and affinity maturation of GC B cells through cellular interactions and cytokine secretion (41–45). In addition, TFH cells also facilitate the differentiation of memory B cells and long-lived plasma cells from GC B cells (21, 22, 46).

TFH cells share similar differentiation processes during different types of immune responses; during the initiation phase of TFH cell differentiation, the expression of some TFs (such as Bcl-6, Ascl2, Maf, and TCF-1) is regulated in certain activated CD4⁺ T cells, which promotes CXCR5 expression (47–51). Next, CXCR5⁺Bcl-6⁺TFH precursor cells migrate to the T-B border zone, where they receive more differentiation signals from activated B cells (52). After this engagement, the reinforced expression of Bcl-6 regulates surface markers, which promote the migration of the TFH cells into GCs, where they provide helper signals to B cells (53, 54). Despite these similarities, TFH cells are also endowed with some unique characteristics for responding to distinct microenvironment associated with different types of microbial infection. Previous studies showed that TFH cells also express lineage-specific TFs like their conventional counterparts when defending against different types of pathogens, such as T-bet, GATA-3, or ROR- γ t in type I, II, or III immune responses, respectively (28, 55–60). The production of IFN- γ , IL4, or IL17 driven by these specific TFs in TFH cells can help B cells switch to the optimal class of antibody to clear the microbes (25, 29, 33, 61–66). However, the extent to which TFH cells rely on these TFs for their differentiation or maintenance is not clear.

The transcription factor T-bet was originally discovered as a lineage marker of TH1 cells because it can establish TH1 differentiation and inhibit polarization of other CD4⁺ T cell subsets such as TH2 or TH17 cells (67–69). Later, it was also found to be extensively expressed by multiple different lymphocyte lineages during type I immune response, including both innate and adaptive immune cell subsets (70–72). For example, T-bet has been reported to promote the early differentiation and terminal maturation of NK cells (73–75). It has also been found that T-bet can promote IFN- γ production in ILCs and $\gamma\delta$ T cells (4, 76, 77). Moreover, T-bet expressed by DCs can enhance their TH1-priming capacity (78). In NKT cells, T-bet can upregulate CD122 levels and promote survival (74, 79). In addition, T-bet is required for optimal terminal differentiation and granzyme B secretion in CD8⁺ T cells (80, 81). Moreover,

T-bet expressed by B cells can promote the survival of memory B cells and enhance IgG2 switching (66, 82). Together, these facts highlight T-bet as the master regulator of type I immune response. In type I immune response, activated antigen-specific CD4⁺ T helper cells mainly differentiate into TH1 and TFH cell subsets (27, 83). Most studies have focused on the role of T-bet in TH1 differentiation and have generally considered T-bet to be a suppressor of TFH differentiation (72, 84–86). However, the exact role of T-bet in the TFH cell response is not well-understood.

In this study, using a combined conditional/inducible knockout system, we investigated the putative role of T-bet in regulating the response of virus-specific TFH cell in acute viral infection. We found the constitutive expression of T-bet in TFH cells during acute viral infection. A great reduction in the magnitude of the TFH cell response was observed when T-bet expression was deficient. Furthermore, microarray analysis showed significant differences in function- and proliferation-related genes between WT and Tbx21^{fl/fl} TFH cells. In addition, TFH and TH1 cells showed different levels of T-bet dependency in their lineage-specific expression patterns. Thus, our findings demonstrate the crucial and specific role of T-bet in type I TFH cell responses, which suggests that modulation of T-bet expression in TFH cells may be a powerful therapeutic method for the treatment of infectious diseases and autoimmune diseases.

MATERIALS AND METHODS

Mice and Treatment

C57BL/6J (CD45.1⁺ and CD45.2⁺), CD4^{cre} transgenic, Ifng^{-/-} and Tbx21^{fl/fl} mice were purchased from Jackson Laboratory. ERT2^{cre} transgenic mice were kindly provided by Yisong Wan (University of North Carolina). SMARTA (CD45.1⁺) mice were a kind gift from Rafi Ahmed (Emory University). All these strains had a C57BL/6J background. All mice were housed and bred under specific-pathogen-free (SPF) conditions. All mouse experiments were performed following the guidelines of the Institutional Animal Care and Use Committees of Army Medical University. All mice were infected/immunized at 6–10 weeks of age. Lymphocytic choriomeningitis virus (LCMV, Armstrong strain) was provided by Rafi Ahmed (Emory University). A total of 2×10^5 plaque-forming units of LCMV (Armstrong strain) were injected intraperitoneally to establish an acute viral infection model in mice. The *Listeria monocytogenes*-expressing LCMV-gp61-80 was created from vector strain 1. A total of 1×10^7 colony-forming units of recombinant bacteria were injected intravenously to establish a mouse bacterial infection model. NP-KLH (100 μ g; N-5060-25; Biosearch Technology) was emulsified 1:1 with Aluminum hydroxide gel (Alum) (21645-51-2; InvivoGen) and was injected subcutaneously to establish a protein immunization model in mice. Tamoxifen (1 mg; T5648; Sigma-Aldrich) was diluted with sunflower oil and injected intraperitoneally into ERT2^{cre}-Tbx21^{fl/fl} or ERT2^{cre}-Tbx21^{fl/fl} mice to induce gene deletion at the indicated timepoints.

Flow Cytometry and Antibodies

Stained cells were analyzed by flow cytometry with a FACS Canto II flow cytometer (BD Bioscience). Flow cytometry data were

analyzed with FlowJo software (Tree Star). LCMV-GP66 tetramer staining was described previously (51). CXCR5 staining has also been described previously (47). Surface staining was performed in PBS containing 2% fetal bovine serum (weight/volume). Staining for intracellular IgG2c, Bcl2, and IFN- γ was performed using a Cytofix/Cytoperm Fixation/Permeabilization Kit (554714; BD Bioscience). Staining for intranuclear TCF-1, T-bet and FOXP3 was performed with a Foxp3/Transcription Factor Staining Buffer Set (00-5523; eBioscience). For intracellular cytokine production analysis, before surface and intracellular staining, cells were stimulated with GP61-80 peptide for 5 h at 37°C, 5% CO₂ in the presence of GolgiPlug (BD Bioscience), GolgiStop (BD Bioscience), and DNase I (Sigma-Aldrich). For *in vivo* incorporation of BrdU, mice were given BrdU (1.5 mg of BrdU in 0.5 ml of DPBS) intraperitoneally 3 h before staining. BrdU staining was performed with a BrdU Flow Kit (559619; BD Bioscience) according to the manufacturer's instructions. Annexin V staining was performed with an Annexin V Apoptosis Detection Kit I (559763; BD Bioscience) according to the manufacturer's instructions. The antibodies and reagents used in flow cytometry staining are listed in **Table S1**.

Enzyme-Linked Immunosorbent Assay

LCMV-specific IgG and IgG2c were titrated with LCMV lysates and the secondary antibodies HRP-conjugated goat anti-mouse IgG (1036-05; SouthernBiotech) and HRP-conjugated goat anti-mouse IgG2c (1078-05; SouthernBiotech) as previously described (87).

Adoptive Cell Transfer

To examine the LCMV-specific TFH cell response, 1×10^6 (for analysis before day 3 or after day 30) or 2×10^5 (for analysis between day 3 and day 30) sorted naïve or retrovirus-transduced CD45.1⁺ SMARTA cells (WT or Tbx21^{-/-}) were adoptively transferred into naïve or infection-matched CD45.2⁺ mice (WT or Tbx21^{-/-}) according to the requirements of the experiments. After being allowed to rest for one day, the cell-transferred hosts were infected intravenously with 2×10^6 plaque-forming units (for analysis at day 3 or earlier) or infected intraperitoneally with 2×10^5 plaque-forming units (for analysis at day 5 or later).

Mixed Bone Marrow Chimera

To determine the intrinsic role of T-bet, bone marrow cells collected from CD45.2⁺ Tbx21^{-/-} mice and CD45.1⁺ WT mice were mixed at a ratio of 3:7 and transferred intravenously into lethally irradiated (5.5 Gy, twice) naïve WT CD45.1⁺ mice (5×10^6 cells/mouse). After at least 8 weeks of bone marrow reconstitution, the recipients were infected with LCMV.

Quantitative RT-PCR

To compare gene expression in LCMV-specific TH1 cells and TFH cells differentiated from naïve WT and Tbx21^{-/-} SMARTA cells, SLAM^{hi}CXCR5⁻ and SLAM^{low}CXCR5⁺ SMARTA cells were sorted from recipient mice and directly lysed with TRIzol LS reagent (10296; Life Technologies). Total RNA was extracted with isopropyl ethanol and reverse-transcribed with a RevertAid H Minus First Strand cDNA Synthesis Kit

(K1632; Thermo Scientific). Quantitative PCR of cDNA was carried out with a QuantiNova SYBR Green PCR Kit (208054; Qiagen) on a CFX96 Touch Real-Time System (Bio-Rad). The sequences of *Tbx21* primers used in RT-qPCR are listed here: *Tbx21* (F)-5' CAATGTGACCCAGATGATCG 3'; *Tbx21*(R)-5' CAATGTGACCCAGATGATCG 3'. Expression was calculated normalized to *Hprt*.

Microarray and Analysis

For the isolation of LCMV-specific TH1 cells and TFH cells, SLAM^{hi}CXCR5⁻ and SLAM^{low}CXCR5⁺ SMARTA cells were sorted from recipient mice adoptively transferred with WT or Tbx21^{-/-} SMARTA cells at day 6 post LCMV infection. For the isolation of naïve CD4⁺ T cells, CD44⁻CD62L⁺CD4⁺ T cells were sorted from naïve WT C57BL/6J mice. The cells were sorted directly into TRIzol LS reagent (10296; Life Technologies). Total RNA was extracted with isopropyl ethanol and submitted to the CapitalBio Corporation for microarray analysis. Gene set enrichment analysis (GSEA) was performed as described previously (88). Clustering analysis was performed and heat maps were constructed using Cluster 3.0 with a hierarchical average linkage method, and the results were visualized using Java TreeView software. Pathway enrichment analysis was performed using KOBAS 3.0 (89).

Immunofluorescence Staining

Spleen tissues were snap frozen in O.C.T. compound (4583; SAKURA) and stored at -80°C until frozen sectioning. The tissues were cut into 10 μ m-thick cryosections and fixed with ice-cold acetone. The sections were rehydrated and blocked with 5% rat serum and 3% BSA with 0.1% Tween and stained with fluorescent-labeled antibodies and reagents, including CD4 (RM4-5; BioLegend), IgD (11-26c.2a; BioLegend), GL7 (GL7; BD Bioscience), and DAPI (R37606; Invitrogen). Images were obtained with an EVOS FL Imaging System (ThermoFisher).

Statistical Analysis

Statistical analysis was performed with Prism 6.0. Differences between groups were analyzed with paired (for bone marrow chimera experiments) or unpaired two-tailed *t*-tests. A *p*-value <0.05 was considered significant.

RESULTS

The Transcription Factor T-Bet Is Selectively Expressed in Type I but not Type II TFH Cells

Previously, it has been reported that T-bet is expressed in mouse TFH cells in LCMV infection model (27), which belongs to type I immune response. However, whether TFH cells express T-bet in other type I immune response models or in type II immune responses is not clear. Thus, we first examined the expression of T-bet in *Listeria monocytogenes* (LM) infection, NP-KLH immunization and LCMV infection models. Based on the expression of CD44 and CXCR5, FOXP3⁻CD4⁺ T cells were divided into three subsets, CD44⁺CXCR5⁺, CD44⁺CXCR5⁻,

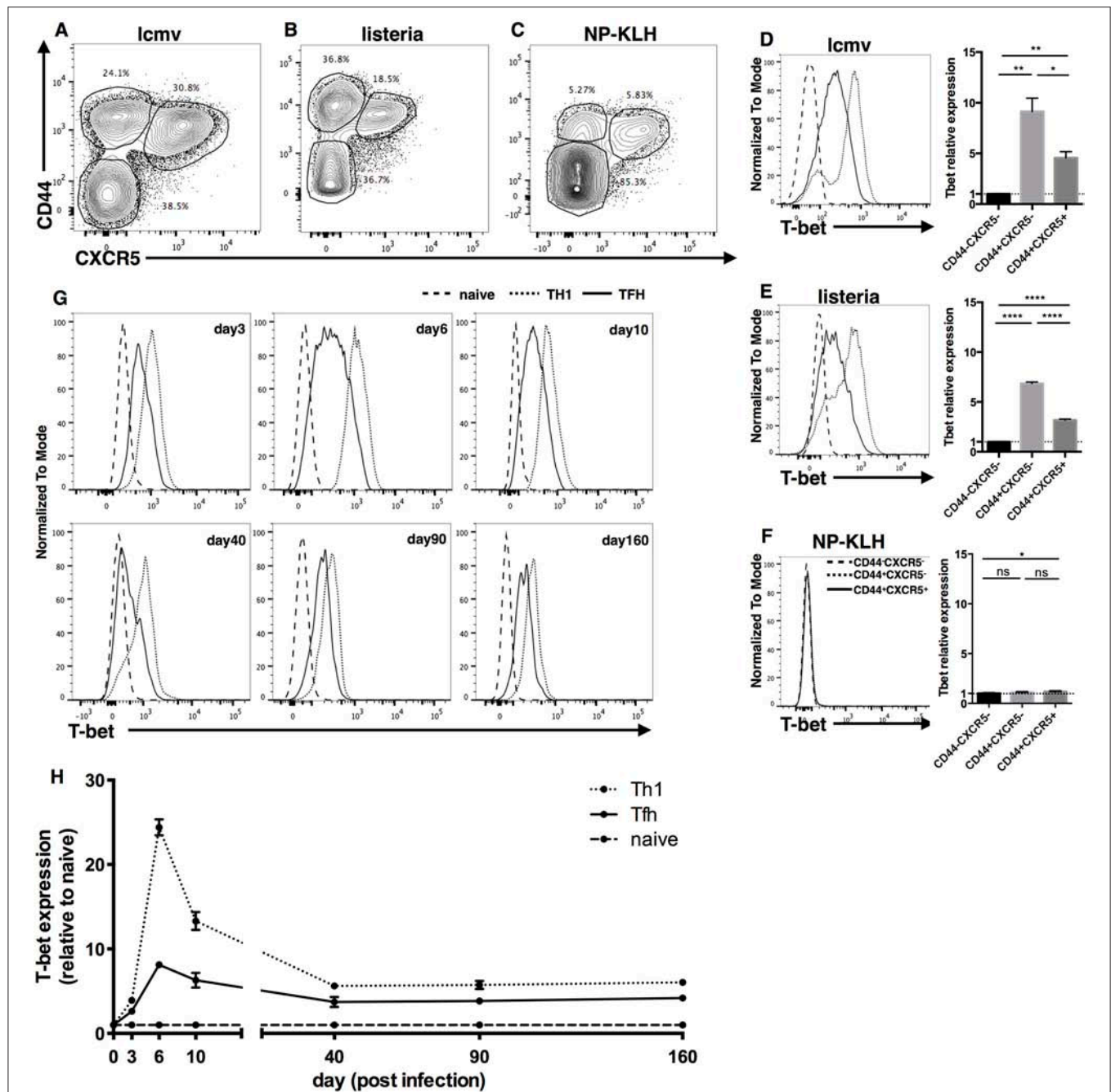


FIGURE 1 | Transcription factor T-bet is selectively expressed in Type I but not Type II TFH cells. **(A–F)** WT C57BL/6 mice were infected with LCMV, LM, or immunized with NP-KLH. Lymphocytes of Spleen (for LCMV and LM infection) or draining lymph nodes (for NP-KLH immunization in alum) were isolated and analyzed for T-bet expression in TFH cells at day 8 post infection/immunization. **(A–C)** Representative flow cytometry of TFH cells (CD44⁺CXCR5⁺), Non-TFH cells (CD44⁺CXCR5⁻) and Naïve CD4⁺ T cells (CD44⁺CXCR5⁻) in LCMV **(A)**, LM **(B)**, infection or NP-KLH **(C)** immunization model. Numbers adjacent to outlined areas indicate percent of each subset in parent subset. **(D–F)** Representative Flow cytometry of T-bet expression in TFH cells, Non-TFH cells and Naïve CD4⁺ T cells (left) and the summary of T-bet expression by calculating the mean fluorescence intensity (MFI) of T-bet in each cell subsets (right) during LCMV **(D)**, LM **(E)** infection, or NP-KLH **(F)** immunization in alum. **(G,H)** WT SMARTA cells (CD45.1⁺) were transferred into WT naïve C57BL/6 mice (CD45.2⁺) and the splenocytes were analyzed for T-bet expression of virus-specific TFH and TH1 cells at day3, 6, 10, 40, 90, and 160 post LCMV infection. **(G)** Representative Flow cytometry of T-bet expression in TFH cells (CD45.1⁺CXCR5⁺), TH1 cells (CD45.1⁺CXCR5⁻) and Naïve CD4⁺ T cells (CD45.2⁺CD44⁺). **(H)** Kinetics of T-bet expression of TFH and TH1 cells by calculating the MFI of T-bet in each subset followed by normalization to the T-bet MFI of Naïve CD4⁺ T cells. ns, not significant; **P* < 0.05, ***P* < 0.01, *****P* < 0.0001 (unpaired two-tailed *t*-test). Data are representative of two independent experiments with 3–5 mice per group (error bars, SEM).

and CD44⁺CXCR5⁺ cells, which were referred to as TFH, non-TFH and naïve CD4⁺ T cells, respectively (Figures 1A–C). At day 8 after immunization, we observed that TFH cells and non-TFH cells generated from the LCMV/LM infection model expressed much higher levels of T-bet than naïve CD4⁺ T cells (Figures 1D,E). In addition, we noticed that TFH cells expressed less T-bet than non-TFH cells in the LCMV/LM infection model (Figures 1D,E), which is consistent with published data (27). However, in the NP-KLH immunization model, there is nearly no detectable T-bet expression in both TFH cells and non-TFH cells (Figure 1F). These data demonstrated that T-bet is selectively expressed in TFH cells derived from type I rather than type II immune responses, suggesting that unlike common transcription factors such as TCF1 or Bcl6, T-bet may be an immune response type-dependent feature of TFH cells.

Next, focusing on the expression of T-bet in type I TFH cells, we investigated the expression kinetics of T-bet in LCMV-specific TFH cells using a SMARTA cell adoptive transfer system. SMARTA cells express LCMV-gp66-specific TCRs, so they can recognize and respond to LCMV and other LCMV-gp66 epitope-carrying microbes (90). We purified naïve ly5.1 SMARTA cells from spleen tissue, transferred them into naïve C57BL/6J recipient mice, and infected the recipient mice with the LCMV strain Armstrong. At different time points post infection, we measured the expression of T-bet in donor SMARTA TH1 and TFH cells. At day 3 post infection, T-bet expression in TFH and TH1 cells was ~2- and 4-fold higher, respectively, than that in naïve CD4⁺ T cells (Figures 1G,H). At day 6 post infection, T-bet expression in TFH and TH1 cells was upregulated to nearly 8- and 24-fold, respectively, compared to that in naïve CD4⁺ T cells (Figures 1G,H). At day 10 post infection, T-bet expression in TFH and TH1 cells had decreased back to levels ~6- and 13-fold higher than those in naïve CD4⁺ T cells (Figures 1G,H). At day 40, 90, and 160 post infection, T-bet expression in TFH and TH1 cells had further decreased and remained ~4- and 6-fold higher, respectively, than that in naïve CD4⁺ T cells (Figures 1G,H). Taken together, these data indicate that TFH and TH1 cells share a dynamic similarity in their T-bet expression patterns: T-bet expression sharply increases in the early effect phase, gradually falls back in the contraction phase, and is stably maintained at a certain level in the memory phase. Meanwhile, consistently lower levels of T-bet were observed in TFH cells than in TH1 cells throughout the entire response.

T-Bet Is Required for TFH Cell Expansion During Acute Viral Infection

To investigate whether T-bet is required for optimal TFH cell responses during acute viral infection, we generated a CD4^{cre}-Tbx21^{fl/fl} strain of mice (called Tbx21^{-/-} mice here) by crossing transgenic CD4^{cre} mice with Tbx21^{fl/fl} mice to selectively knock out the Tbx21 gene (encoding the T-bet protein) in T cells. These Tbx21^{-/-} mice showed normal T cell development *in vivo* (Figures S1A,B). At day 8 post LCMV Armstrong infection, the RT-qPCR and flow cytometry results showed that TH1 and TFH cells from Tbx21^{-/-} mice did not express T-bet (Figures 2A,B). In addition, we observed a significant decrease in the frequency

and number of polyclonal CD44⁺CXCR5⁺ TFH cells in the spleens of Tbx21^{-/-} mice (Figure 2C). To determine whether this phenotype was caused by deficient clonal expansion or abnormal differentiation, we used the gp66 tetramer to measure antigen-specific CD4⁺ T cells. The results showed that fewer gp66-specific CD4⁺ T cells were present in Tbx21^{-/-} mice (Figure 2D), indicating that clonal expansion of gp66-specific Tbx21^{-/-} CD4⁺ T cells was heavily affected. Consistent with the decreased number of gp66-specific CD4⁺ T cells, the number of gp66-specific TFH cells was also greatly decreased in Tbx21^{-/-} mice (Figure 2E). Furthermore, we observed a mild increase in the frequency of gp66-specific Tbx21^{-/-} TFH cells (Figure 2E), which was consistent with a report that T-bet inhibits TFH cell differentiation *in vitro* (84). Together, these data suggest that T-bet is required for TFH cell response mainly by promoting clonal expansion during acute viral infection.

Optimal Germinal Center Response Requires T-Bet Expression in TFH Cells

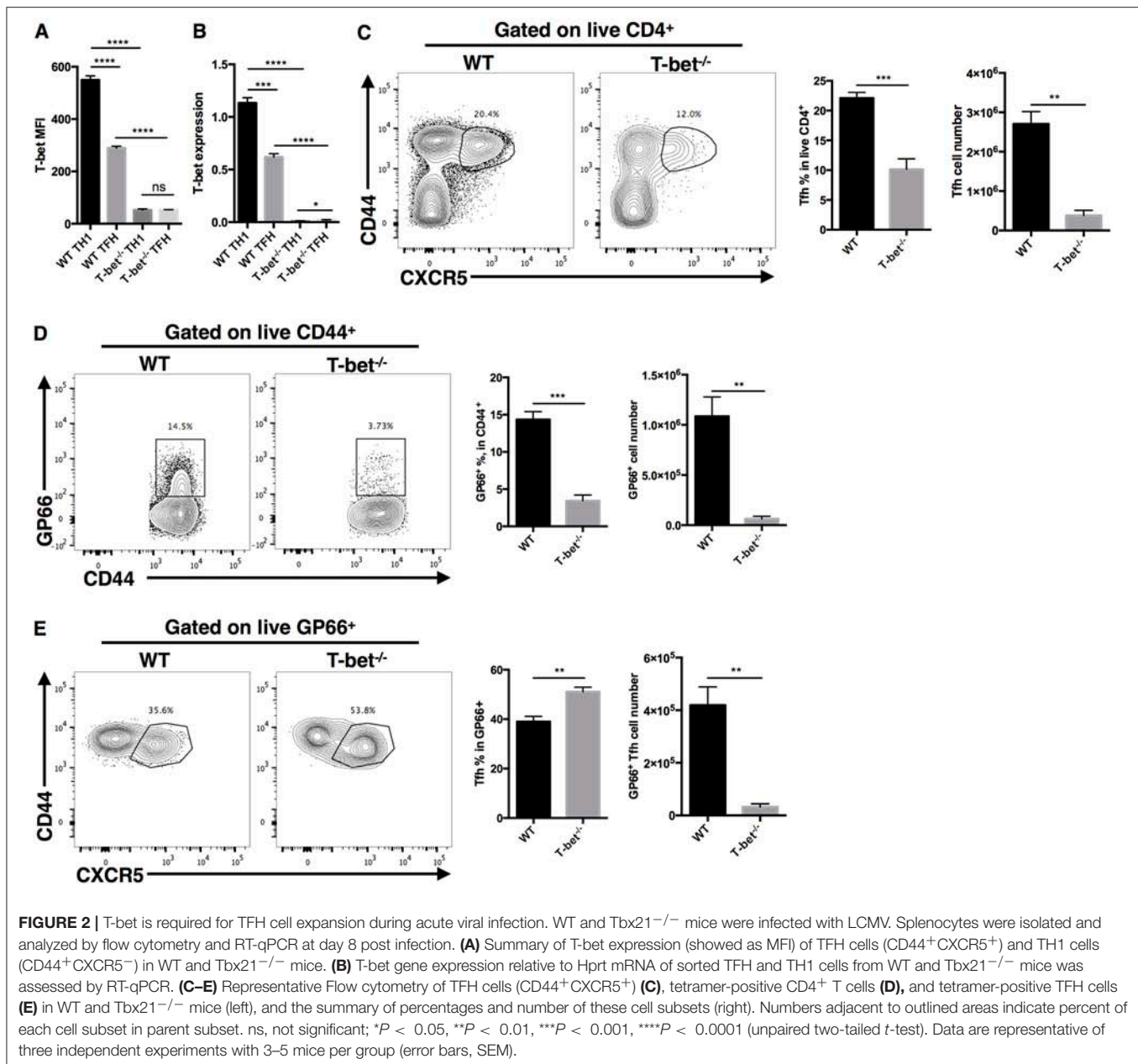
Based on the critical role of TFH cells in “helping” GC response, we next investigated whether T-bet deficiency in TFH cells would influence the germinal center response. At day 8 post LCMV Armstrong infection, we observed severely affected GC formation in the spleens of Tbx21^{-/-} mice (Figure 3A). Loss of T-bet expression in TFH cells strongly reduced the frequency of GC B cells and plasma cells (Figures 3B,C). In addition, Tbx21^{-/-} mice showed less IgG2c class switching in plasma cells (Figure 3D) as well as much lower LCMV-specific IgG and subtype IgG2c titers in serum (Figures 3E,F) than WT mice. These data further verified the vital role of T-bet in promoting the TFH cell response and antibody IgG2 class switching.

T-Bet Is not Required for Type II TFH Cell Response

The observation of a compromised type I TFH response in Tbx21^{-/-} mice during acute viral infection led us to investigate whether T-bet plays an important role in type II TFH cell response. Thus, we tested the TFH and GC responses of Tbx21^{-/-} mice in a protein immunization model. At day 8 post NP-KLH immunization, we observed similar frequencies and numbers of TFH cells in Tbx21^{-/-} mice and control mice (Figure S2A). In addition, we did not find any reductions in the frequency or number of GC B cells as well as plasma cells in Tbx21^{-/-} mice (Figures S2B,C). These results are consistent with the observation that T-bet is not expressed in CD4⁺ T cells during type II immune response. Together with the crucial role of T-bet in regulating type I TFH cell response during acute viral infection, these results confirm that T-bet is an environmentally specific regulator of type I TFH cell response.

T-Bet Promotes TFH Cell Expansion in a T Cell Intrinsic Manner

In the CD4^{cre}-induced Tbx21 knockout system, both CD4⁺ and CD8⁺ T cells lost their capacity to express T-bet. In addition, a deficient GC response might reciprocally amplify the impairment of the TFH cell response in Tbx21^{-/-} mice. To further clarify the

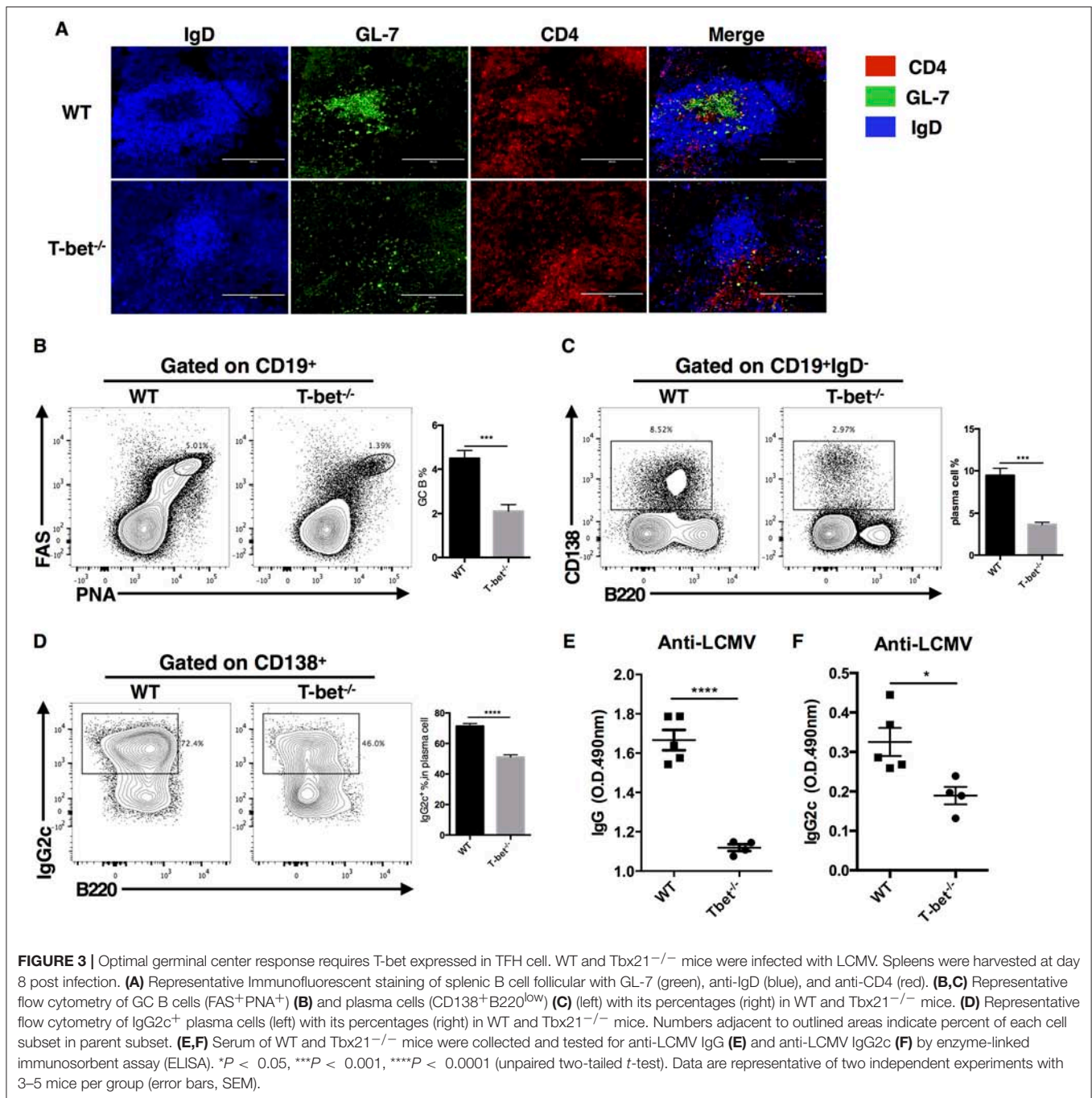


cell-intrinsic role of T-bet in regulating the TFH cell response, we set up bone marrow chimeras by reconstituting lethally irradiated WT (ly5.1⁺) recipient mice with a 3:7 ratio mixture of bone marrow cells from *Tbx21*^{-/-} (ly5.2⁺) and WT (ly5.1⁺) donor mice, respectively (Figure 4A). Chimera mice were infected with LCMV Armstrong after successful bone marrow reconstitution (Figures S3A,B). At day 8 post infection, we still observed a largely decreased frequency of gp66-specific CD4⁺ T cells and polyclonal TFH cells in *Tbx21*^{-/-} (ly5.2⁺) mice compared to control mice (Figures 4B,C). Similar to what we found in *Tbx21*^{-/-} mice, the frequency of gp66-specific TFH cells was slightly increased in *Tbx21*^{-/-} cells of chimera mice (Figure 4D). Moreover, the *Tbx21*^{-/-}: WT ratios of polyclonal TFH cells,

gp66-specific CD4⁺ T cells and gp66-specific TFH cells were markedly decreased relative to that of total CD4⁺ T cells (Figures 4B–D). These data confirmed the intrinsic role of T-bet in regulating the TFH cell response during acute viral infection.

T-Bet Promotes TFH Cell Maintenance by Regulating Proliferation and Apoptosis

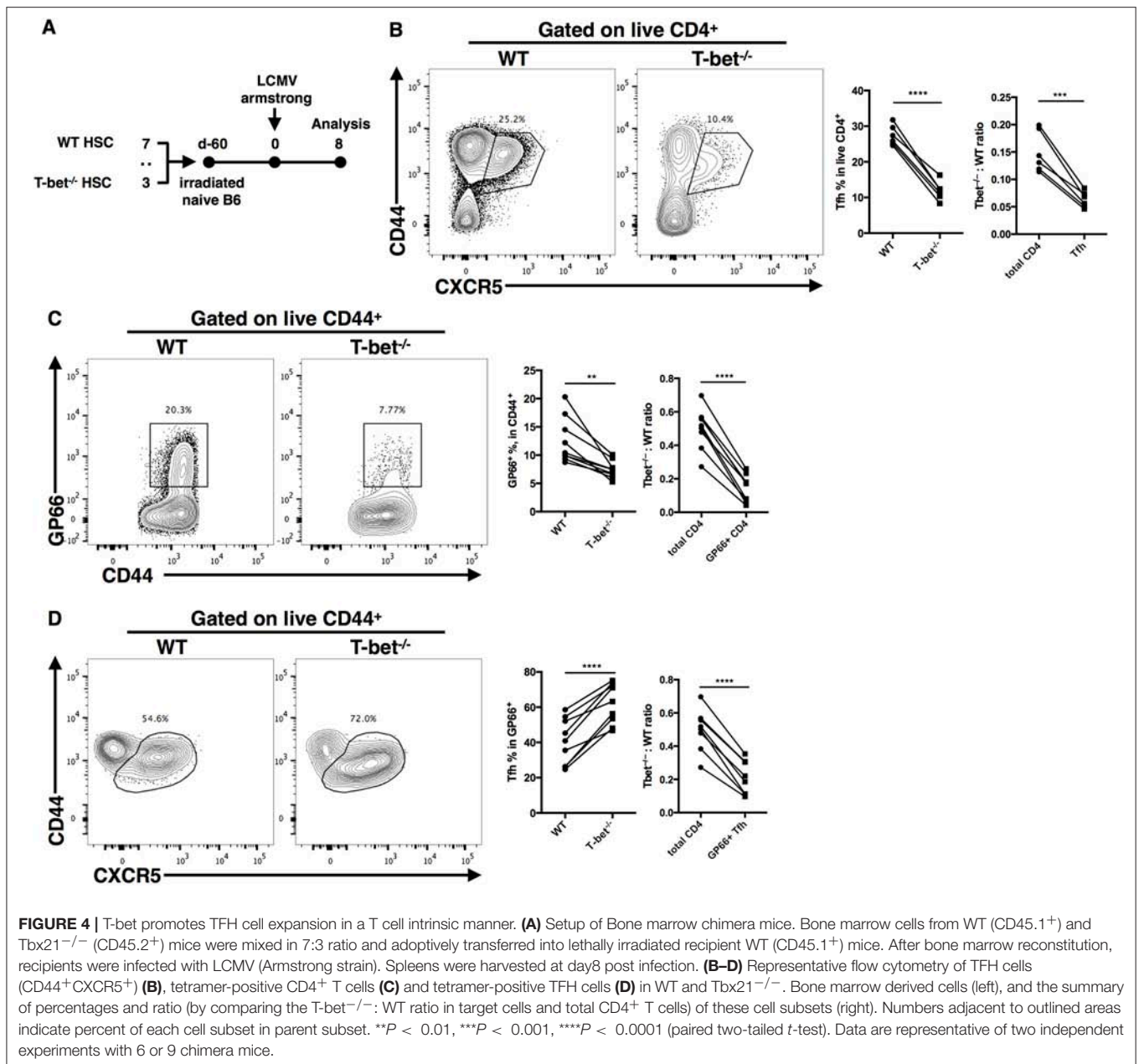
It was clear that the deficiency of the TFH cell response in *Tbx21*^{-/-} mice was mainly caused by the greatly reduced magnitude of the TFH cell response. To investigate the kinetics of virus-specific TFH cell expansion in *Tbx21*^{-/-} mice, we transferred the same number of WT or *Tbx21*^{-/-} SMARTA cells into naïve recipient mice and then infected host mice with LCMV



Armstrong. From day 2 post infection, we detected a continuous slightly higher frequency of TFH cells in the *Tbx21*^{-/-} SMARTA group than in the WT group (**Figures 5A,B**). At day 2 and day 5 post infection, we did not observe any differences in the numbers of *Tbx21*^{-/-} and WT SMARTA TFH cells (**Figures 5A,B**). To our surprise, the number of *Tbx21*^{-/-} SMARTA TFH cells decreased sharply at day8 post infection (**Figures 5A,B**). Besides, the reduction in the virus-specific TFH cell population in *Tbx21*^{-/-} mice might not have been caused by impaired early activation of CD4⁺ T cells (**Figures S4A,B**). Taken together,

the results suggest the possibility that the loss of TFH cells in *Tbx21*^{-/-} mice was mainly caused by reduced maintenance at the late phase of the anti-viral immune response.

To more carefully investigate the influence of T-bet on TFH cell maintenance at the late phase of infection, we generated ERT2^{cre}-*Tbx21*^{fl/fl} mice (i*Tbx21*^{-/-}) by crossing *Tbx21*^{fl/fl} mice with ERT2^{cre} transgenic mice, in which *Tbx21* gene knockout could be induced by tamoxifen treatment. We treated mice with tamoxifen at 1–3 days before or 5–7 days after LCMV infection to induce T-bet deletion before or after TFH cell commitment,



respectively. Under both of these circumstances, we observed a lower abundance of TFH cells in iTbx21^{-/-} mice at day 9 post infection (Figures 5C,D). In addition, iTbx21^{-/-} CD4⁺ T cells were purified and adoptively transferred into infection-matched recipient mice at day 7 post LCMV infection (Figure 5E). After 3 days of tamoxifen or vehicle administration (days 8–10), we observed a decreased number of donor TFH cells in mice treated with tamoxifen than in control mice at day 14 post infection (Figure 5F). These results suggest that T-bet is required for the TFH cell response even after TFH commitment.

Furthermore, to investigate the sharp decrease in the number of Tbx21^{-/-} SMARTA TFH cells at the late effector phase, we sorted WT and Tbx21^{-/-} SMARTA TFH cells from

recipient mice at day 6 post LCMV Armstrong infection and adoptively transferred a 1:1 ratio mixture of WT and Tbx21^{-/-} SMARTA TFH cells into infection-matched mice (Figure 5G). At day 9 post infection, we observed that the ratio of WT and Tbx21^{-/-} SMARTA TFH cells had changed to ~4:1 (Figure 5H). Taken together, these results indicated that intrinsic expression of T-bet is essential for TFH maintenance at the late effector phase.

Next, to gain insight into the reason for the reduction in the TFH cell population, we measured the proliferation and apoptosis of SMARTA TFH cells. At day 2 post infection, we observed even higher proliferation and expression of the survival marker Bcl2 but comparable apoptosis in Tbx21^{-/-}

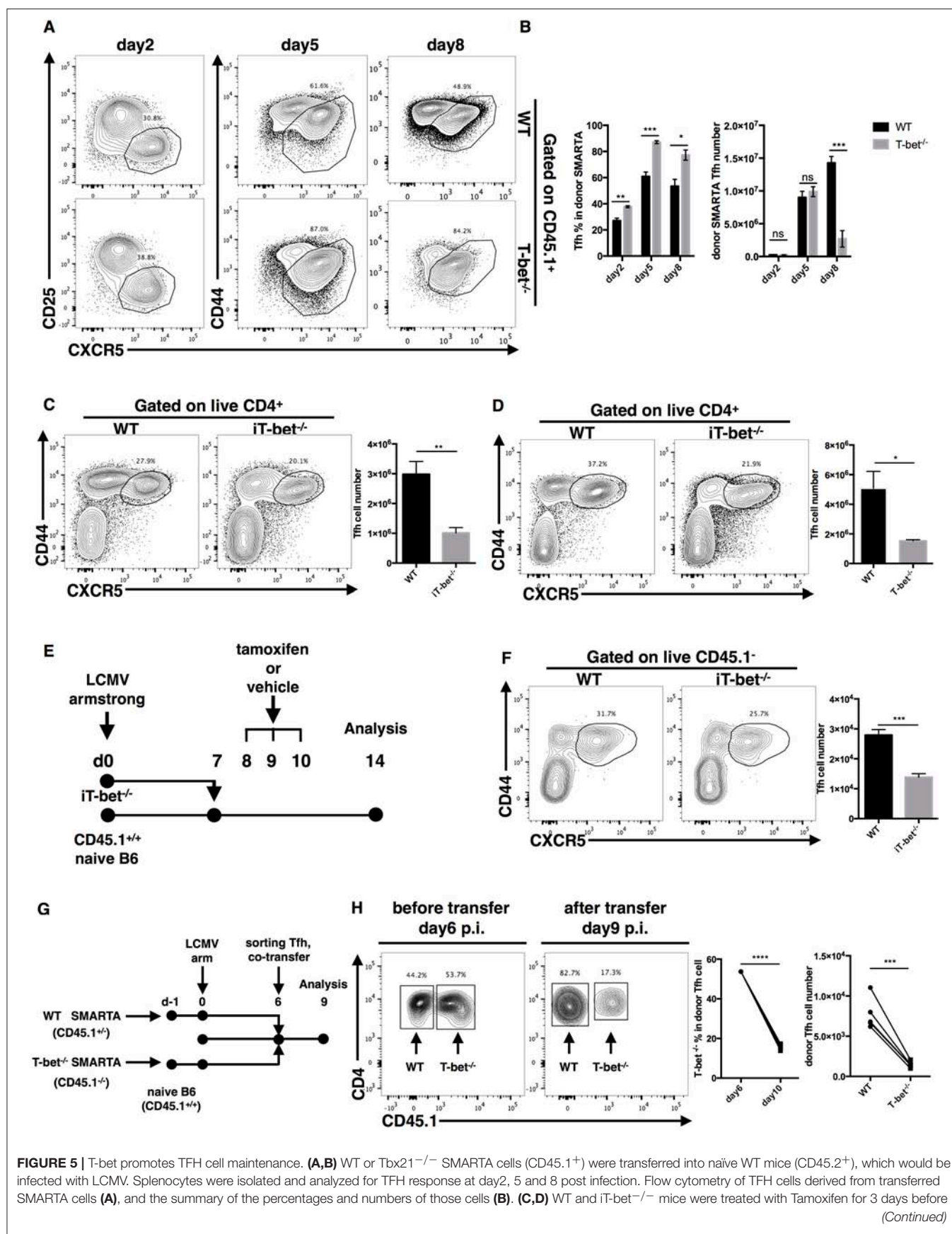


FIGURE 5 | or after LCMV infection. Spleens were harvested at day 9 post infection. **(C)** Flow cytometry of TFH cells (left) with its number (right) in mice treat with Tamoxifen before infection (day -3 to -1). **(D)** Flow cytometry of TFH cells (left) with its number (right) in mice treat with Tamoxifen after infection (day 5-7). **(E)** Setup of CD4⁺ T cell transfer experiment. CD4⁺ T cells were purified from spleens of iT-bet^{-/-} mice (CD45.2⁺) without Tamoxifen treatment and adoptively transferred into infection-matched recipient mice (CD45.1⁺). At day 8-10 post infection, recipient mice were treated with Tamoxifen or vehicle. Spleens were harvested at day 14 post infection. **(F)** Representative flow cytometry of TFH cells (left) and summary of TFH cell number (right) in Tamoxifen treated mice (iTbx21^{-/-}) and vehicle treated mice (WT) was showed here. **(G)** Setup of TFH co-transfer experiments. WT SMARTA cells (CD45.1⁺CD45.2⁺) and Tbx21^{-/-} SMARTA cells (CD45.1⁻CD45.2⁺) were transferred into naïve B6 mice (CD45.1⁺CD45.2⁻) separately before infecting recipients with LCMV. At day 6 post infection, WT SMARTA TFH cells and Tbx21^{-/-} SMARTA TFH cells were FACS sorted and mixed (about 1:1), then co-transferred into infection-matched B6 mice (CD45.1⁺CD45.2⁻). Spleens were harvested and analyzed for transferred TFH cells at day 9 post infection. **(H)** Flow cytometry of sorted SMARTA TFH cells before (day 6 p.i.) and after (day 9 p.i.) co-transfer (left). The percentage of Tbx21^{-/-} SMARTA TFH cells in total donor TFH cells and the number of WT and Tbx21^{-/-} SMARTA TFH cells at day 9 post infection were summarized (right). Numbers adjacent to outlined areas in **(A,C,D,F,H)** indicate percent of each cell subset in parent subset. ns, not significant; **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001 (unpaired **(B-D,F)** or paired **(H)** two-tailed *t*-test). Data in **(C,D,F)** are representative of two independent experiments with 3-5 mice per group (error bars, SEM).

SMARTA TFH cells compared to WT cells (**Figures 6A-C**). However, at day 5 post infection, the proliferation rate of Tbx21^{-/-} SMARTA TFH cells dropped quickly to a significantly lower level than that of WT cells (**Figure 6A**), which was consistent with the lower Bcl2 expression in Tbx21^{-/-} SMARTA TFH cells (**Figure 6C**). At day 8 post infection, higher apoptosis rates and lower Bcl2 expression were found in Tbx21^{-/-} SMARTA TFH cells than in WT cells (**Figures 6B,C**). These results suggested that T-bet controls the TFH cell maintenance ultimately by promoting proliferation at the mid phase and inhibiting apoptosis at the late effector phase.

T-Bet Dependency of the TFH and TH1 Cell Transcriptomes

To investigate the molecular mechanisms regulated by T-bet in TFH and TH1 cell response, we sorted WT and Tbx21^{-/-} SMARTA TFH and TH1 cells from recipient mice at day 6 post LCMV Armstrong infection after adoptive transfer, as well as naïve mice-derived CD4⁺ T cells, for gene expression profile analysis. The gene expression patterns differed greatly between WT and Tbx21^{-/-} cell population at the genome-wide level (**Figures 7A,B**). We observed 822 upregulated and 899 downregulated genes in Tbx21^{-/-} TH1 cells relative to WT TH1 cells (**Figure S5A**). Accordingly, we identified 151 upregulated and 367 downregulated genes in Tbx21^{-/-} TFH cells (**Figure S5B**). Among these differentially expressed genes, 103-up and 223-down regulated genes were shared by Tbx21^{-/-} TFH and TH1 cells (**Figure 7C**). Besides, PANTHER pathway enrichment analysis of the changed genes in Tbx21^{-/-} TFH and TH1 cells also showed similar enrichment in many important pathways, such as DNA replication, Apoptosis, and Interferon-gamma signaling pathway, which account for the impaired maintenance of Tbx21^{-/-} TFH cells (**Figures S5C,D**). To further figure out the downstream factors involved in the maintenance of differentiated TFH and TH1 cells controlled by T-bet, we did the gene set enrichment analysis focusing on cell proliferation and survival and found a reduction to a similar extent in an array of proliferation and survival relevant genes, such as *Ccna2*, *Ccnb2*, *Aurkb*, *E2f1*, *E2f7*, and *E2f8* in both Tbx21^{-/-} TH1 and TFH cells compared to their WT counterpart (**Figure 7D**), highlighting the shared regulatory pathway important for both TFH and TH1

proliferation and survival that is likely imprinted by the same Type-I microenvironment (58).

Despite these similarities, bioinformatics analysis of the microarray data also observed many differences in the dependency of T-bet in TFH and TH1 cell development. Principal component analysis (PCA) showed that after T-bet deletion, the gene expression profile of Tbx21^{-/-} TH1 cells shifted toward that of TFH cells to some extent (**Figure 7B**). Then, we selected sets of genes that were upregulated in TFH cells (TFH-UP) compared with non-TFH cells or upregulated in TH1 cells (TH1-UP) compared with TFH cells based on both published data (91) (GEO accession code GSE21379) and our microarray data (GEO accession code GSE122931). Gene set enrichment analysis (GSEA) of TH1 cells showed that the TH1-UP gene set was enriched in WT TH1 cells, whereas the TFH-UP gene set was enriched in Tbx21^{-/-} TH1 cells, which suggested to some extent that the gene-expression pattern of Tbx21^{-/-} TH1 cells lost TH1 signature and change to TFH signature (**Figure 7E**). Similar to that of TH1 cells, GSEA of TFH cells showed that the TH1-UP gene set was enriched in WT TFH cells (**Figure 7F**). However, different from what we have observed in TH1 cells, genes in the TFH-UP gene set were enriched in WT TFH cells much more than in Tbx21^{-/-} TFH cells, which suggested that the gene-expression pattern of Tbx21^{-/-} TFH cells lost TH1 signature but also lost part of its TFH signature (**Figure 7F**). Through further assessed 48 genes known to be associated with T cell differentiation or function, we found a distinct T-bet dependency of the TFH and TH1 cell gene-expression pattern (**Figure 7G**). In TH1 cells, We found that the transcription levels of a set of important Th1 lineage associated genes like *Il12rb2* (92) *Cxcr3*, *Prdm1*, *Gzmb*, *Gzmk*, *Gzma*, *Cx3cr1*, and *Ifng* were significantly decreased in Tbx21^{-/-} Th1 cells than in WT TH1 cells. We also observed *Prdm1* expression was lower in Tbx21^{-/-} TH1 cells than in WT TH1 cells. In addition, the expression of *Foxp3*, *Gata3*, and *Rorc*, which are essential for Treg, TH2, and TH17 cell differentiation, respectively, was higher in Tbx21^{-/-} TH1 cells than in WT TH1 cells (**Figure 7G**). On the other hand, the abundances of TFH lineage-specification associated genes, including *Tox2*, *Id3*, *Bhlhe40*, *Il6st*, *Il6ra*, and *Tcf7* were much increased in Tbx21^{-/-} TFH cells (**Figure 7G**), at least in part explaining the differential role of T-bet in regulating the program of TH1 and TFH differentiation. We compared the expression

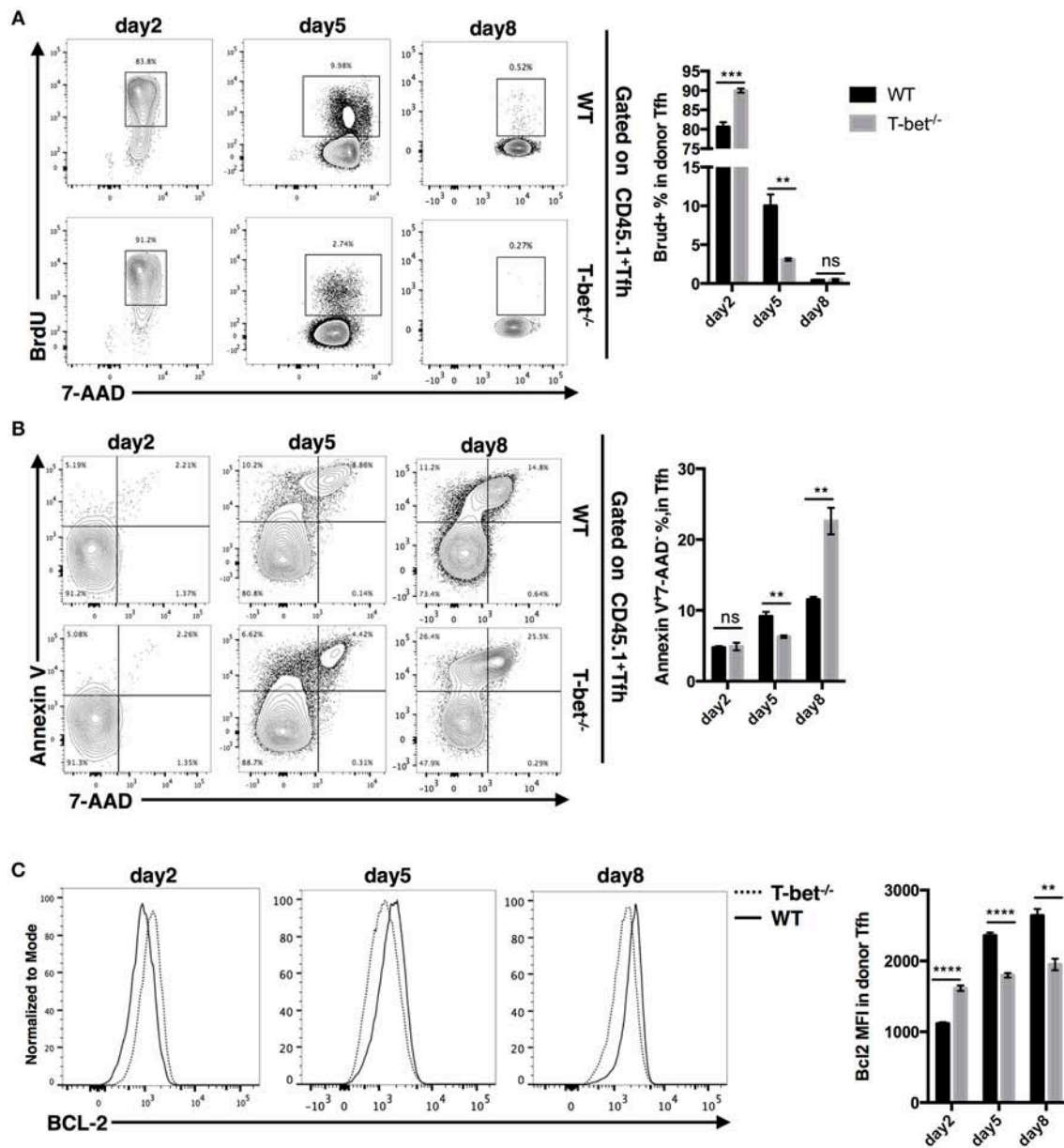


FIGURE 6 | T-bet regulates TFH cell proliferation and apoptosis. WT or Tbx21^{-/-} SMARTA cells (CD45.1⁺) were transferred into naïve WT mice (CD45.2⁺), which would be infected with LCMV. Splenocytes were isolated and analyzed for TFH response at day 2, 5, and 8 post infection. **(A)** Flow cytometry of BrdU incorporation in TFH cells (left), and summary of BrdU-incorporated cells percentage (right). Numbers adjacent to outlined areas indicate percent of each cell subset in parent subset. **(B)** Flow cytometry of Annexin-V and 7-AAD staining in TFH cells (left), and summary of Annexin-V⁺ 7-AAD⁺ percentage (right). Numbers in quadrants indicate percent cells in each. **(C)** Flow cytometry of BCL-2 expression in TFH cells (left), and the summary of Bcl-2 expression (showed as MFI) of TFH cells. ns, not significant; ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001 (unpaired two-tailed *t*-test). Data are representative of two independent experiments with 3–5 mice per group (error bars, SEM).

of *Batf4* (93) and *Irf4* in WT and Tbx21^{-/-} TFH cells. As expected, we observed the dramatically decrease expression of *Batf4* and *Irf4* in Tbx21^{-/-} TFH cells. Besides, the significant lower expression of *Icos* (94) was also detected in Tbx21^{-/-} TFH cells compared to that in WT counterparts (**Figure 7G**). Whereas, expression of other TFH cell-relevant genes like *Prdm1*, *Bcl6*, and *Cxcr5* was not significantly influenced by

T-bet deletion (**Figure 7G**). The imbalanced impact of T-bet deletion on TH1 and TFH cells may interpret the mildly higher frequency of TFH cells than TH1 cells in antigen-specific CD4⁺ T cells that we observed (**Figures 2E, 4D, 5A**). Together, these data indicate both similarities and differences in transcriptome dependency on T-bet in TFH and TH1 cells during acute viral infection.

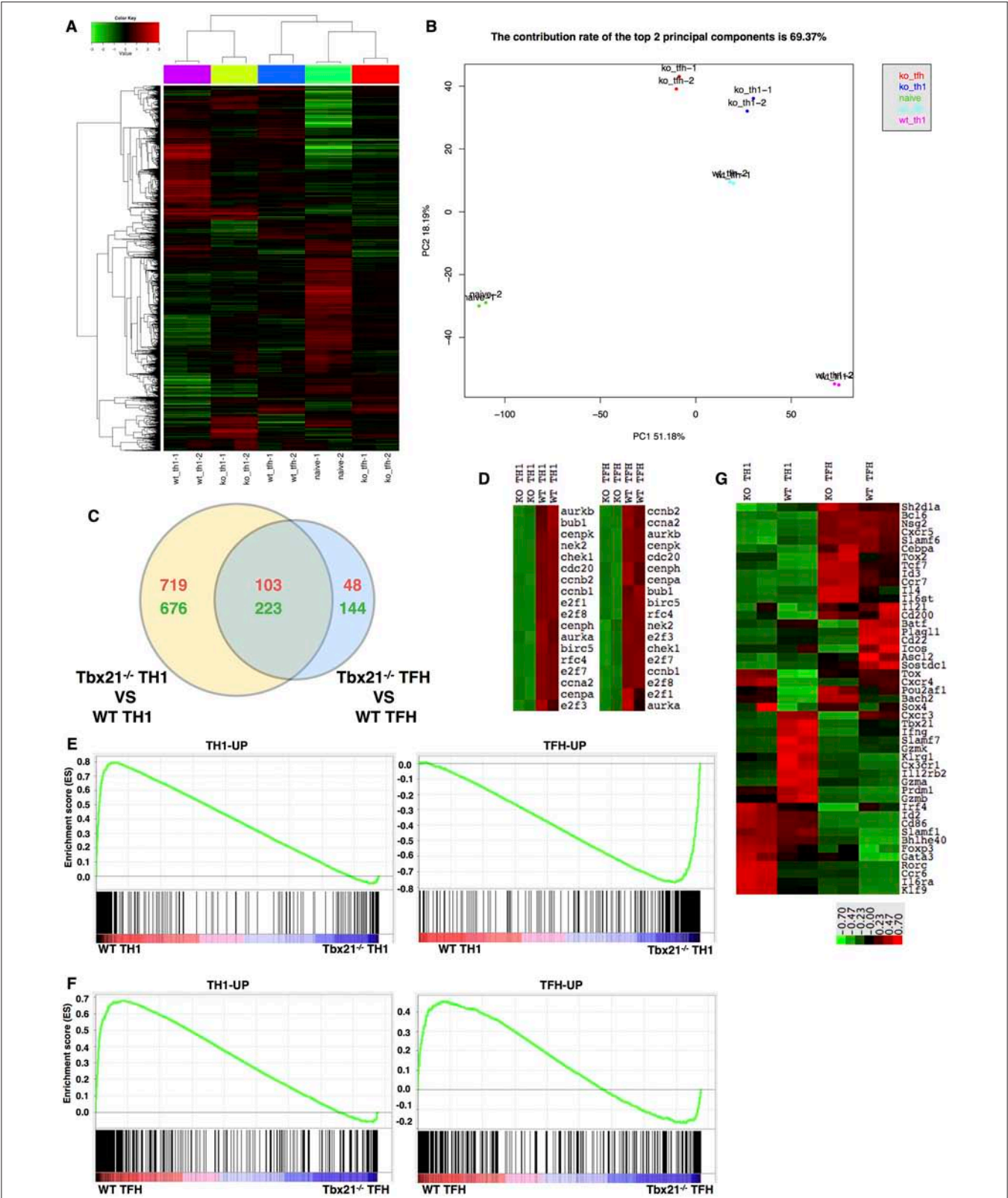


FIGURE 7 | T-bet dependency of the TFH and TH1 cell transcriptomes. **(A)** Clustering of genes based on their expression in WT TH1, WT TFH, Tbx21^{-/-} TH1, and Tbx21^{-/-} TFH cells. **(B)** Principal component analysis (PCA) of genes in the five cell populations listed in **(A)**. **(C)** Venn diagram comparing the differentially expressed genes in TH1 and TFH cells. The red numbers represent upregulated genes, and the green numbers represent downregulated genes. **(D)** Heat map of genes related to TH1 and TFH cells. **(E)** Enrichment scores (ES) for TH1-UP and TFH-UP genes. **(F)** Enrichment scores (ES) for TH1-UP and TFH-UP genes. (Continued)

FIGURE 7 | cell proliferation in WT TH1, Tbx21^{-/-} TH1, WT TFH, and Tbx21^{-/-} TFH cells. **(E)** GSEA of WT and Tbx21^{-/-} TH1 genes showing gene sets upregulated in TH1 cells relative to TFH cells (left) and gene sets upregulated in TFH cells relative to TH1 cells (right). **(F)** GSEA of WT and Tbx21^{-/-} TFH genes showing gene sets upregulated in TH1 cells relative to TFH cells (left) and gene sets upregulated in TFH cells relative to TH1 cells (right). The gene sets used in GSEA include published data (GEO accession code GSE21379) and our microarray data (GEO accession code GSE122931). **(G)** Heat map of genes related to T cell differentiation and function in WT TH1, Tbx21^{-/-} TH1, WT TFH, and Tbx21^{-/-} TFH cells.

IFN- γ as a Candidate Downstream Target of T-Bet in Regulating TFH Cell Expansion

As a direct target of T-bet, it has been reported that IFN- γ could promote clonal expansion and survival of CD4⁺ T cells (95). Microarray analysis also showed that the interferon-gamma signaling pathway was severely impaired in TFH cells after T-bet deletion. In addition, we observed largely decreased IFN- γ production in *ex vivo* recalled Tbx21^{-/-} TFH cells (Figures 8A,B). To investigate if IFN- γ could regulate TFH cell expansion, we compared the TFH cell responses in Tbx21^{-/-} and Ifng^{-/-} mice during LCMV Armstrong infection. At day 8 post infection, we found that the frequency and number of TFH cells were decreased in both Tbx21^{-/-} and Ifng^{-/-} mice (Figure 8C). In addition, T-bet expression was not affected by IFN- γ deletion (Figure 8D). Although not sufficient, these results suggest that IFN- γ might be a candidate target of T-bet in regulating TFH cell expansion.

DISCUSSION

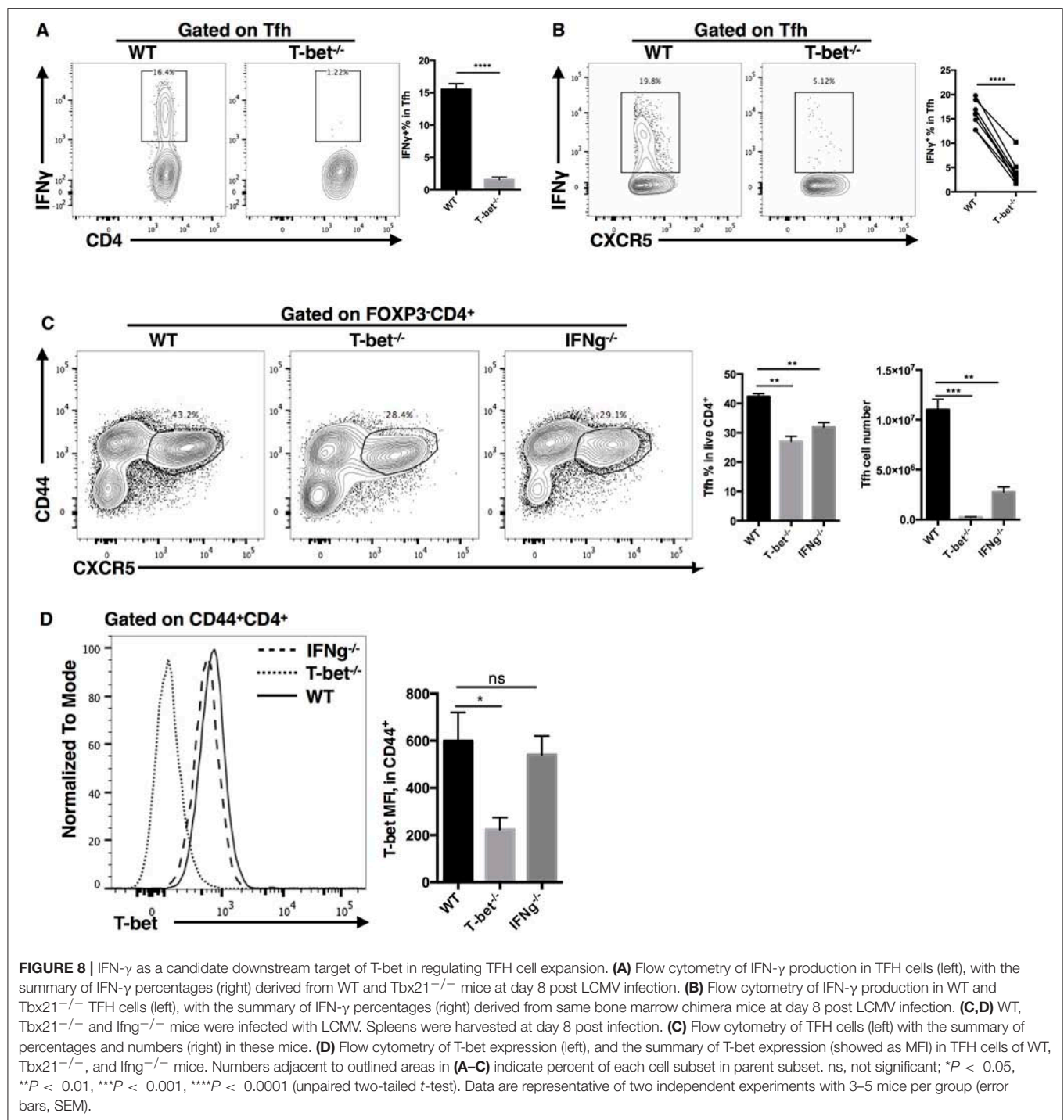
Follicular helper T cells (TFH cells) play critical roles in type I, II, and III immune responses, but how TFH cells adapt to different environments has remained largely unknown. In this study, we identified a key link between the transcription factor T-bet and type I TFH cell response during acute viral infection. We found that T-bet is specifically expressed in TFH cells originating from type I but not type II immune response. Tbx21^{-/-} mice exhibited significant deficiency in the TFH cell response during acute viral infection. We observed a greatly decreased magnitude of the TFH cell response in Tbx21^{-/-} mice compared to WT mice, although a slightly increased ratio of TFH cells was observed. Based on these results, we concluded that T-bet is required for optimal type I TFH cell response.

In addition to LCMV infection, type I TFH cells have also been discovered as TH1-biased TFH cells in simian immunodeficiency virus infection and TH1-polarized TFH cells in malaria infection (96, 97). Type I TFH cells not only express Bcl6, CXCR5, IL21, and PD-1 but also coexpress CXCR3 and IFN- γ . The secreted IFN- γ can help antibodies from B cells switch to the IgG2a/c class, which is essential for efficient elimination of viruses and other pathogens. Similar to the case in TH1 cells, the expression of TH1-associated molecules in type I TFH cells is also induced by the transcription factor T-bet. In addition, our research demonstrated the crucial role of T-bet in promoting TFH cell proliferation and maintenance. Based on the specialized role of type I TFH cells in defending against intracellular pathogens, we propose that this Th1-like effector TFH population be named TFH1.

During TH1 differentiation, it has been reported that T-bet attenuates the TFH cell-like phenotype in the late phase of TH1 specification by repressing the expression of Bcl6 and other molecules associated with TFH cell development (84). In addition, T-bet has been found to inhibit Tcf7 expression by directly binding with the Tcf7 gene promoter and suppress Bcl6 function by physically interacting with the Bcl6 protein (85, 86, 98). These *in vitro* results suggest that T-bet uses multiple mechanisms to inhibit TFH differentiation. However, evidence supporting the role of T-bet in regulating the TFH phenotype is not sufficient at the *in vivo* level. In our study, we clearly showed that the maintenance of TFH at the later effector phase is sharply impaired after T-bet deletion even though mildly increased early TFH differentiation was observed. Additionally, the constitutive expression of T-bet in type I TFH cells may suppress early TFH generation but sustain the clonal expansion of TFH cells at the late stage. Thorough understanding of the distinct role of T-bet in type I TFH cells at different stages necessitates further investigation in the future.

Notably, we did not observe any T-bet expression in type II TFH cells during protein immunization. Accordingly, Tbx21^{-/-} mice showed normal TFH and GC responses during protein immunization. These results remind us that, unlike Bcl6 or Blimp1, the transcription factor T-bet is not a fundamental regulator of “all-weather” TFH cell responses under natural conditions. In other words, T-bet is a type I TFH cell-specific regulator, suggesting that diverse transcription factors are required for optimal TFH cell responses in different environments.

In addition, our microarray analysis results showed that there are not only many similar but also many different important changes in the TFH and TH1 cell transcriptomes that occur in a T-bet-dependent manner. On the one hand, Tbx21^{-/-} TFH cells share many altered genes with TH1 cells, including genes enriched in signaling pathways involved in DNA replication, apoptosis and interferon-gamma signaling. On the other hand, many TFH differentiation-related genes were altered in different directions and to different degrees in Tbx21^{-/-} TH1 and TFH cells. Four possible mechanisms might be involved in this scenario. First, some genes are regulated by T-bet in a redundant way, which means that the regulatory role of T-bet may be unnecessary if these genes have already been up- or downregulated by other transcription factors. Second, differences in chromatin accessibility between TFH and TH1 cells would lead to differences in the binding affinity of T-bet. Third, by interacting with different transcription factors, T-bet could differentially regulate gene expression in TFH and TH1 cells. Fourth, the post transcriptional modification of T-bet is different in TFH and TH1 cells, which might result in different or even



opposite regulatory functions at the same gene loci. Further studies are needed to explore the exact mechanism underlying the contradictory effects of T-bet in regulating the development of TH1 and TFH cells.

Overall, this study revealed that T-bet, although slightly inhibiting TFH differentiation, mainly supports type I TFH cell response by promoting cell proliferation and apoptotic intervention to maintain the TFH cell response at the late

effector phase during acute viral infection. These findings provide important insights into the transcription factor-mediated regulation of the environmental suitability of TFH cells.

DATA AVAILABILITY

The datasets generated for this study can be found in Gene Expression Omnibus, GSE122931.

ETHICS STATEMENT

All mouse experiments were performed following the guidelines of the Institutional Animal Care and Use Committees (IACUCs) of Army Medical University. The protocols were approved by the IACUCs.

AUTHOR CONTRIBUTIONS

PW, YC, YuW, and LY designed and supervised the study. PW, YW, LuX, MX, JW, QH, BL, XC, LiX, SY, and YH performed experiments. PW wrote the manuscript with YW. QB and RH helped with analysis. All authors read and approved the manuscript.

FUNDING

This study was supported by grants from the National Natural Science Foundation of China (No. 31825011 to LY; No. 31700774 to LiX) and Open research fund of State Key Laboratory of Veterinary Biotechnology (SKLVB2018XX to LY) and the

National Key Research Development Plan (No. 2016YFA0502202 to LY) and the National Science and Technology Major Project of China during the 13th Five-year Plan (2018ZX1030 2104 to YC).

ACKNOWLEDGMENTS

We thank Yisong Wan (University of North Carolina) for kindly providing us ERT2^{cre} transgenic mice; We thank Rafi Ahmed (Emory University) for generous providing us LCMV Armstrong virus and SMARTA transgenic mice. We also thank CapitalBio Corporation (Beijing, China) for performing microarray experiments and data analysis, and we thank the core facility center of Army Medical University for cell sorting.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2019.00606/full#supplementary-material>

REFERENCES

- Annunziato F, Romagnani C, Romagnani S. The 3 major types of innate and adaptive cell-mediated effector immunity. *J Allergy Clin Immunol.* (2015) 135:626–35. doi: 10.1016/j.jaci.2014.11.001
- Klose CSN, Flach M, Mohle L, Rogell L, Hoyler T, Ebert K, et al. Differentiation of type 1 ILCs from a common progenitor to all helper-like innate lymphoid cell lineages. *Cell.* (2014) 157:340–56. doi: 10.1016/j.cell.2014.03.030
- Sun JC, Lanier LL. NK cell development, homeostasis and function: parallels with CD8(+) T cells. *Nat Rev Immunol.* (2011) 11:645–57. doi: 10.1038/nri3044
- Bernink JH, Peters CP, Munneke M, te Velde AA, Meijer SL, Weijer K, et al. Human type 1 innate lymphoid cells accumulate in inflamed mucosal tissues. *Nat Immunol.* (2013) 14:221–9. doi: 10.1038/ni.2534
- Mosmann TR, Cherwinski H, Bond MW, Giedlin MA, Coffman RL. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J Immunol.* (1986) 136:2348–57.
- Farrar JD, Asnagli H, Murphy KM. T helper subset development: roles of instruction, selection, and transcription. *J Clin Invest.* (2002) 109:431–5. doi: 10.1172/JCI0215093
- Mosmann TR, Sad S, Krishnan L, Wegmann TG, Guilbert LJ, Belosevic M. Differentiation of subsets of CD4+ and CD8+ T cells. *Ciba Found Symp.* (1995) 195:42–50. discussion-50–4.
- Carter LL, Murphy KM. Lineage-specific requirement for signal transducer and activator of transcription (Stat)4 in interferon gamma production from CD4(+) versus CD8(+) T cells. *J Exp Med.* (1999) 189:1355–60. doi: 10.1084/jem.189.8.1355
- Price AE, Liang HE, Sullivan BM, Reinhardt RL, Easley CJ, Erle DJ, et al. Systemically dispersed innate IL-13-expressing cells in type 2 immunity. *Proc Natl Acad Sci USA.* (2010) 107:11489–94. doi: 10.1073/pnas.1003988107
- Fort MM, Cheung J, Yen D, Li J, Zurawski SM, Lo S, et al. IL-25 induces IL-4, IL-5, and IL-13 and Th2-associated pathologies *in vivo*. *Immunity.* (2001) 15:985–95. doi: 10.1016/S1074-7613(01)00243-6
- Walker JA, McKenzie AN. Development and function of group 2 innate lymphoid cells. *Curr Opin Immunol.* (2013) 25:148–55. doi: 10.1016/j.coi.2013.02.010
- Romagnani S. Lymphokine production by human T cells in disease states. *Annu Rev Immunol.* (1994) 12:227–57. doi: 10.1146/annurev.iy.12.040194.001303
- Maggi E, Giudizi MG, Biagiotti R, Annunziato F, Manetti R, Piccinni MP, et al. Th2-like CD8+ T cells showing B cell helper function and reduced cytolytic activity in human immunodeficiency virus type 1 infection. *J Exp Med.* (1994) 180:489–95. doi: 10.1084/jem.180.2.489
- Spits H, Artis D, Colonna M, Diefenbach A, Di Santo JP, Eberl G, et al. Innate lymphoid cells—a proposal for uniform nomenclature. *Nat Rev Immunol.* (2013) 13:145–9. doi: 10.1038/nri3365
- Eberl G, Marmon S, Sunshine MJ, Rennett PD, Choi Y, Littman DR. An essential function for the nuclear receptor RORgamma(t) in the generation of fetal lymphoid tissue inducer cells. *Nat Immunol.* (2004) 5:64–73. doi: 10.1038/ni1022
- Harrington LE, Hatton RD, Mangan PR, Turner H, Murphy TL, Murphy KM, et al. Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. *Nat Immunol.* (2005) 6:1123–32. doi: 10.1038/ni1254
- Acosta-Rodriguez EV, Rivino L, Geginat J, Jarrossay D, Gattorno M, Lanzavecchia A, et al. Surface phenotype and antigenic specificity of human interleukin 17-producing T helper memory cells. *Nat Immunol.* (2007) 8:639–46. doi: 10.1038/ni1467
- Annunziato F, Cosmi L, Santarlasci V, Maggi L, Liotta F, Mazzinghi B, et al. Phenotypic and functional features of human Th17 cells. *J Exp Med.* (2007) 204:1849–61. doi: 10.1084/jem.20070663
- Intlekofer AM, Banerjee A, Takemoto N, Gordon SM, Dejong CS, Shin H, et al. Anomalous type 17 response to viral infection by CD8+ T cells lacking T-bet and eomesodermin. *Science.* (2008) 321:408–11. doi: 10.1126/science.1159806
- Yen HR, Harris TJ, Wada S, Grosso JE, Getnet D, Goldberg MV, et al. Tc17 CD8 T cells: functional plasticity and subset diversity. *J Immunol.* (2009) 183:7161–8. doi: 10.4049/jimmunol.0900368
- Breitfeld D, Ohl L, Kremmer E, Ellwart J, Sallusto F, Lipp M, et al. Follicular B helper T cells express CXCR chemokine receptor 5, localize to B cell follicles, and support immunoglobulin production. *J Exp Med.* (2000) 192:1545–52. doi: 10.1084/jem.192.11.1545
- Schaerli P, Willmann K, Lang AB, Lipp M, Loetscher P, Moser B. CXCR chemokine receptor 5 expression defines follicular homing T cells with B cell helper function. *J Exp Med.* (2000) 192:1553–62. doi: 10.1084/jem.192.11.1553
- Fahey LM, Wilson EB, Elsaesser H, Fistonich CD, McGavern DB, Brooks DG. Viral persistence redirects CD4 T cell differentiation toward T follicular helper cells. *J Exp Med.* (2011) 208:987–99. doi: 10.1084/jem.20101773

24. Harker JA, Lewis GM, Mack L, Zuniga EI. Late interleukin-6 escalates T follicular helper cell responses and controls a chronic viral infection. *Science*. (2011) 334:825–9. doi: 10.1126/science.1208421
25. Boyden AW, Legge KL, Waldschmidt TJ. Pulmonary infection with influenza A virus induces site-specific germinal center and T follicular helper cell responses. *PLoS ONE*. (2012) 7:e40733. doi: 10.1371/journal.pone.0040733
26. Feng J, Hu X, Guo H, Sun X, Wang J, Xu L, et al. Patients with chronic hepatitis C express a high percentage of CD4(+)CXCR5(+) T follicular helper cells. *J Gastroenterol*. (2012) 47:1048–56. doi: 10.1007/s00535-012-0568-1
27. Hale JS, Youngblood B, Latner DR, Mohammed AU, Ye L, Akondy RS, et al. Distinct memory CD4+ T cells with commitment to T follicular helper- and T helper 1-cell lineages are generated after acute viral infection. *Immunity*. (2013) 38:805–17. doi: 10.1016/j.immuni.2013.02.020
28. Glatman Zaretsky A, Taylor JJ, King IL, Marshall FA, Mohrs M, Pearce EJ. T follicular helper cells differentiate from Th2 cells in response to helminth antigens. *J Exp Med*. (2009) 206:991–9. doi: 10.1084/jem.20090303
29. King IL, Mohrs M. IL-4-producing CD4+ T cells in reactive lymph nodes during helminth infection are T follicular helper cells. *J Exp Med*. (2009) 206:1001–7. doi: 10.1084/jem.20090313
30. Kemeny DM. The role of the T follicular helper cells in allergic disease. *Cell Mol Immunol*. (2012) 9:386–9. doi: 10.1038/cmi.2012.31
31. Kamekura R, Shigehara K, Miyajima S, Jitsukawa S, Kawata K, Yamashita K, et al. Alteration of circulating type 2 follicular helper T cells and regulatory B cells underlies the comorbid association of allergic rhinitis with bronchial asthma. *Clin Immunol*. (2015) 158:204–11. doi: 10.1016/j.clim.2015.02.016
32. Meli AP, Fontes G, Leung Soo C, King IL. T Follicular helper cell-derived IL-4 is required for IgE production during intestinal helminth infection. *J Immunol*. (2017) 199:244–52. doi: 10.4049/jimmunol.1700141
33. Ding R, Gao W, He Z, Wang H, Conrad CD, Dinney CM, et al. Overrepresentation of Th1- and Th17-like follicular helper T cells in coronary artery disease. *J Cardiovasc Transl Res*. (2015) 8:503–5. doi: 10.1007/s12265-015-9662-0
34. Smith C, Buhlmann JE, Wang X, Bartlett A, Lim B, Barrington RA. CD275-Independent IL-17-producing T follicular helper-like cells in lymphopenic autoimmune-prone mice. *J Immunol*. (2016) 196:4935–46. doi: 10.4049/jimmunol.1402193
35. Yang Y, Zhang M, Ye Y, Ma S, Fan L, Li Z. High frequencies of circulating Tfh-Th17 cells in myasthenia gravis patients. *Neurol Sci*. (2017) 38:1599–1608. doi: 10.1007/s10072-017-3009-3
36. Moser B, Ebert L. Lymphocyte traffic control by chemokines: follicular B helper T cells. *Immunol Lett*. (2003) 85:105–12. doi: 10.1016/S0165-2478(02)00233-X
37. Hardtke S, Ohl L, Forster R. Balanced expression of CXCR5 and CCR7 on follicular T helper cells determines their transient positioning to lymph node follicles and is essential for efficient B-cell help. *Blood*. (2005) 106:1924–31. doi: 10.1182/blood-2004-11-4494
38. Rasheed AU, Rahn HP, Sallusto F, Lipp M, Muller G. Follicular B helper T cell activity is confined to CXCR5(hi)ICOS(hi) CD4 T cells and is independent of CD57 expression. *Eur J Immunol*. (2006) 36:1892–903. doi: 10.1002/eji.200636136
39. Fazilleau N, Mark L, McHeyzer-Williams LJ, McHeyzer-Williams MG. Follicular helper T cells: lineage and location. *Immunity*. (2009) 30:324–35. doi: 10.1016/j.immuni.2009.03.003
40. Moser B. CXCR5, the defining marker for follicular B helper T (TFH) Cells. *Front Immunol*. (2015) 6:296. doi: 10.3389/fimmu.2015.00296
41. Vinuesa CG, Tangye SG, Moser B, Mackay CR. Follicular B helper T cells in antibody responses and autoimmunity. *Nat Rev Immunol*. (2005) 5:853–65. doi: 10.1038/nri1714
42. Bryant VL, Ma CS, Avery DT, Li Y, Good KL, Corcoran LM, et al. Cytokine-mediated regulation of human B cell differentiation into Ig-secreting cells: predominant role of IL-21 produced by CXCR5+ T follicular helper cells. *J Immunol*. (2007) 179:8180–90. doi: 10.4049/jimmunol.179.12.8180
43. King C, Tangye SG, Mackay CR. T follicular helper (TFH) cells in normal and dysregulated immune responses. *Annu Rev Immunol*. (2008) 26:741–66. doi: 10.1146/annurev.immunol.26.021607.090344
44. Nurieva RI, Chung Y, Hwang D, Yang XO, Kang HS, Ma L, et al. Generation of T follicular helper cells is mediated by interleukin-21 but independent of T helper 1, 2, or 17 cell lineages. *Immunity*. (2008) 29:138–49. doi: 10.1016/j.immuni.2008.05.009
45. Vogelzang A, McGuire HM, Yu D, Sprent J, Mackay CR, King C. A fundamental role for interleukin-21 in the generation of T follicular helper cells. *Immunity*. (2008) 29:127–37. doi: 10.1016/j.immuni.2008.06.001
46. Haynes NM. Follicular associated T cells and their B-cell helper qualities. *Tissue Antigens*. (2008) 71:97–104. doi: 10.1111/j.1399-0039.2007.00995.x
47. Johnston RJ, Poholek AC, DiToro D, Yusuf I, Eto D, Barnett B, et al. Bcl6 and Blimp-1 are reciprocal and antagonistic regulators of T follicular helper cell differentiation. *Science*. (2009) 325:1006–10. doi: 10.1126/science.1175870
48. Kroenke MA, Eto D, Locci M, Cho M, Davidson T, Haddad EK, et al. Bcl6 and Maf cooperate to instruct human follicular helper CD4 T cell differentiation. *J Immunol*. (2012) 188:3734–44. doi: 10.4049/jimmunol.1103246
49. Liu X, Chen X, Zhong B, Wang A, Wang X, Chu F, et al. Transcription factor achaete-scute homologue 2 initiates follicular T-helper-cell development. *Nature*. (2014) 507:513–8. doi: 10.1038/nature12910
50. Andris F, Denanglaire S, Anciaux M, Hercor M, Hussein H, Leo O. The Transcription Factor c-Maf promotes the differentiation of follicular helper T cells. *Front Immunol*. (2017) 8:480. doi: 10.3389/fimmu.2017.00480
51. Xu L, Cao Y, Xie Z, Huang Q, Bai Q, Yang X, et al. The transcription factor TCF-1 initiates the differentiation of T(FH) cells during acute viral infection. *Nat Immunol*. (2015) 16:991–9. doi: 10.1038/ni.3229
52. Nurieva RI, Chung Y, Martinez GJ, Yang XO, Tanaka S, Matskevitch TD, et al. Bcl6 mediates the development of T follicular helper cells. *Science*. (2009) 325:1001–5. doi: 10.1126/science.1176676
53. Yu D, Rao S, Tsai LM, Lee SK, He Y, Sutcliffe EL, et al. The transcriptional repressor Bcl-6 directs T follicular helper cell lineage commitment. *Immunity*. (2009) 31:457–68. doi: 10.1016/j.immuni.2009.07.002
54. Baumjohann D, Okada T, Ansel KM. Cutting Edge: distinct waves of BCL6 expression during T follicular helper cell development. *J Immunol*. (2011) 187:2089–92. doi: 10.4049/jimmunol.1101393
55. Ryg-Cornejo V, Ioannidis LJ, Ly A, Chiu CY, Tellier J, Hill DL, et al. Severe malaria infections impair germinal center responses by inhibiting T follicular helper cell differentiation. *Cell Rep*. (2016) 14:68–81. doi: 10.1016/j.celrep.2015.12.006
56. Fang D, Cui K, Mao K, Hu G, Li R, Zheng M, et al. Transient T-bet expression functionally specifies a distinct T follicular helper subset. *J Exp Med*. (2018) 215:2705–15. doi: 10.1084/jem.20180927
57. Ma X, Nakayama S, Kubo S, Sakata K, Yamagata K, Miyazaki Y, et al. Expansion of T follicular helper-T helper 1 like cells through epigenetic regulation by signal transducer and activator of transcription factors. *Ann Rheum Dis*. (2018) 77:1354–61. doi: 10.1136/annrheumdis-2017-212652
58. Weinstein JS, Laidlaw BJ, Lu Y, Wang JK, Schulz VP, Li N, et al. STAT4 and T-bet control follicular helper T cell development in viral infections. *J Exp Med*. (2018) 215:337–55. doi: 10.1084/jem.20170457
59. Hercor M, Anciaux M, Denanglaire S, Debusson D, Leo O, Andris F. Antigen-presenting cell-derived IL-6 restricts the expression of GATA3 and IL-4 by follicular helper T cells. *J Leukoc Biol*. (2017) 101:5–14. doi: 10.1189/jlb.1HI1115-511R
60. Schmitt N, Liu Y, Benteibibel SE, Munagala I, Bourdery L, Venuprasad K, et al. The cytokine TGF-beta co-opts signaling via STAT3-STAT4 to promote the differentiation of human TFH cells. *Nat Immunol*. (2014) 15:856–65. doi: 10.1038/ni.2947
61. Reinhardt RL, Liang HE, Locksley RM. Cytokine-secreting follicular T cells shape the antibody repertoire. *Nat Immunol*. (2009) 10:385–93. doi: 10.1038/ni.1715
62. Lee SK, Silva DG, Martin JL, Pratama A, Hu X, Chang PP, et al. Interferon-gamma excess leads to pathogenic accumulation of follicular helper T cells and germinal centers. *Immunity*. (2012) 37:880–92. doi: 10.1016/j.immuni.2012.10.010
63. Luthje K, Kallies A, Shimohakamada Y, Belz GT, Light A, Tarlinton DM, et al. The development and fate of follicular helper T cells defined by an IL-21 reporter mouse. *Nat Immunol*. (2012) 13:491–8. doi: 10.1038/ni.2261
64. Wong MT, Chen J, Narayanan S, Lin W, Anicete R, Kiaang HT, et al. Mapping the Diversity of follicular helper T cells in human blood and tonsils using high-dimensional mass cytometry analysis. *Cell Rep*. (2015) 11:1822–33. doi: 10.1016/j.celrep.2015.05.022

65. Vijayanand P, Seumois G, Simpson LJ, Abdul-Wajid S, Baumjohann D, Panduro M, et al. Interleukin-4 production by follicular helper T cells requires the conserved IL4 enhancer hypersensitivity site V. *Immunity*. (2012) 36:175–87. doi: 10.1016/j.immuni.2011.12.014
66. Peng SL, Szabo SJ, Glimcher LH. T-bet regulates IgG class switching and pathogenic autoantibody production. *Proc Natl Acad Sci USA*. (2002) 99:5545–50. doi: 10.1073/pnas.082114899
67. Szabo SJ, Kim ST, Costa GL, Zhang X, Fathman CG, Glimcher LH. A novel transcription factor, T-bet, directs Th1 lineage commitment. *Cell*. (2000) 100:655–69. doi: 10.1016/S0092-8674(00)80702-3
68. Hwang ES, Szabo SJ, Schwartzberg PL, Glimcher LH. T helper cell fate specified by kinase-mediated interaction of T-bet with GATA-3. *Science*. (2005) 307:430–3. doi: 10.1126/science.1103336
69. Oestreich KJ, Weinmann AS. T-bet employs diverse regulatory mechanisms to repress transcription. *Trends Immunol*. (2012) 33:78–83. doi: 10.1016/j.it.2011.10.005
70. Kallies A, Good-Jacobson KL. Transcription factor T-bet orchestrates lineage development and function in the immune system. *Trends Immunol*. (2017) 38:287–97. doi: 10.1016/j.it.2017.02.003
71. Lazarevic V, Glimcher LH. T-bet in disease. *Nat Immunol*. (2011) 12:597–606. doi: 10.1038/ni.2059
72. Lazarevic V, Glimcher LH, Lord GM. T-bet: a bridge between innate and adaptive immunity. *Nat Rev Immunol*. (2013) 13:777–89. doi: 10.1038/nri3536
73. Gordon SM, Chaix J, Rupp LJ, Wu J, Madera S, Sun JC, et al. The transcription factors T-bet and Eomes control key checkpoints of natural killer cell maturation. *Immunity*. (2012) 36:55–67. doi: 10.1016/j.immuni.2011.11.016
74. Townsend MJ, Weinmann AS, Matsuda JL, Salomon R, Farnham PJ, Biron CA, et al. T-bet regulates the terminal maturation and homeostasis of NK and Valpha14i NKT cells. *Immunity*. (2004) 20:477–94. doi: 10.1016/S1074-7613(04)00076-7
75. Werneck MB, Lugo-Villarino G, Hwang ES, Cantor H, Glimcher LH. T-bet plays a key role in NK-mediated control of melanoma metastatic disease. *J Immunol*. (2008) 180:8004–10. doi: 10.4049/jimmunol.180.12.8004
76. Klose CS, Kiss EA, Schwierzeck V, Ebert K, Hoyler T, d'Hargues Y, et al. A T-bet gradient controls the fate and function of CCR6-RORgammat+ innate lymphoid cells. *Nature*. (2013) 494:261–5. doi: 10.1038/nature11813
77. Chen L, He W, Kim ST, Tao J, Gao Y, Chi H, et al. Epigenetic and transcriptional programs lead to default IFN-gamma production by gammadelta T cells. *J Immunol*. (2007) 178:2730–6. doi: 10.4049/jimmunol.178.5.2730
78. Lugo-Villarino G, Maldonado-Lopez R, Possemato R, Penaranda C, Glimcher LH. T-bet is required for optimal production of IFN-gamma and antigen-specific T cell activation by dendritic cells. *Proc Natl Acad Sci USA*. (2003) 100:7749–54. doi: 10.1073/pnas.1332767100
79. Matsuda JL, Zhang Q, Ndonye R, Richardson SK, Howell AR, Gapin L. T-bet concomitantly controls migration, survival, and effector functions during the development of Valpha14i NKT cells. *Blood*. (2006) 107:2797–805. doi: 10.1182/blood-2005-08-3103
80. Cruz-Guilloty F, Pipkin ME, Djuretic IM, Levanon D, Lotem J, Lichtenheld MG, et al. Runx3 and T-box proteins cooperate to establish the transcriptional program of effector CTLs. *J Exp Med*. (2009) 206:51–9. doi: 10.1084/jem.20081242
81. Takemoto N, Intlekofer AM, Northrup JT, Wherry EJ, Reiner SL. Cutting Edge: IL-12 inversely regulates T-bet and eomesodermin expression during pathogen-induced CD8+ T cell differentiation. *J Immunol*. (2006) 177:7515–9. doi: 10.4049/jimmunol.177.11.7515
82. Wang NS, McHeyzer-Williams LJ, Okitsu SL, Burris TP, Reiner SL, McHeyzer-Williams MG. Divergent transcriptional programming of class-specific B cell memory by T-bet and RORalpha. *Nat Immunol*. (2012) 13:604–11. doi: 10.1038/ni.2294
83. Marshall HD, Chandeale A, Jung YW, Meng H, Poholek AC, Parish IA, et al. Differential expression of Ly6C and T-bet distinguish effector and memory Th1 CD4(+) cell properties during viral infection. *Immunity*. (2011) 35:633–46. doi: 10.1016/j.immuni.2011.08.016
84. Nakayama S, Kanno Y, Takahashi H, Jankovic D, Lu KT, Johnson TA, et al. Early Th1 cell differentiation is marked by a Tfh cell-like transition. *Immunity*. (2011) 35:919–31. doi: 10.1016/j.immuni.2011.11.012
85. Oestreich KJ, Huang AC, Weinmann AS. The lineage-defining factors T-bet and Bcl-6 collaborate to regulate Th1 gene expression patterns. *J Exp Med*. (2011) 208:1001–13. doi: 10.1084/jem.20102144
86. Oestreich KJ, Mohn SE, Weinmann AS. Molecular mechanisms that control the expression and activity of Bcl-6 in TH1 cells to regulate flexibility with a TFH-like gene profile. *Nat Immunol*. (2012) 13:405–11. doi: 10.1038/ni.2242
87. Rasheed MA, Latner DR, Aubert RD, Gourley T, Spolski R, Davis CW, et al. Interleukin-21 is a critical cytokine for the generation of virus-specific long-lived plasma cells. *J Virol*. (2013) 87:7737–46. doi: 10.1128/JVI.00063–13
88. Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, et al. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad Sci USA*. (2005) 102:15545–50. doi: 10.1073/pnas.0506580102
89. Wu J, Mao X, Cai T, Luo J, Wei L. KOBAS server: a web-based platform for automated annotation and pathway identification. *Nucleic Acids Res*. (2006) 34:W720–4. doi: 10.1093/nar/gkl167
90. Oxenius A, Bachmann MF, Zinkernagel RM, Hengartner H. Virus-specific MHC-class II-restricted TCR-transgenic mice: effects on humoral and cellular immune responses after viral infection. *Eur J Immunol*. (1998) 28:390–400. doi: 10.1002/(SICI)1521-4141(199801)28:01<390::AID-IMMU390>3.0.CO;2-O
91. Yusuf I, Kageyama R, Monticelli L, Johnston RJ, Ditoro D, Hansen K, et al. Germinal center T follicular helper cell IL-4 production is dependent on signaling lymphocytic activation molecule receptor (CD150). *J Immunol*. (2010) 185:190–202. doi: 10.4049/jimmunol.09.03505
92. Xiao L, Jia L, Zhang Y, Yu S, Wu X, Yang B, et al. Human IL-21+IFN-gamma+CD4+ T cells in nasal polyps are regulated by IL-12. *Sci Rep*. (2015) 5:12781. doi: 10.1038/srep12781
93. Ise W, Kohyama M, Schraml BU, Zhang T, Schwer B, Basu U, et al. The transcription factor BATF controls the global regulators of class-switch recombination in both B cells and T cells. *Nat Immunol*. (2011) 12:536–43. doi: 10.1038/ni.2037
94. Stone EL, Pepper M, Katayama CD, Kerdiles YM, Lai CY, Emslie E, et al. ICOS coreceptor signaling inactivates the transcription factor FOXO1 to promote Tfh cell differentiation. *Immunity*. (2015) 42:239–51. doi: 10.1016/j.immuni.2015.01.017
95. Reed JM, Branigan PJ, Bamezai A. Interferon gamma enhances clonal expansion and survival of CD4+ T cells. *J Interferon Cytokine Res*. (2008) 28:611–22. doi: 10.1089/jir.2007.0145
96. Velu V, Mylvaganam GH, Gangadhara S, Hong JJ, Iyer SS, Gumber S, et al. Induction of Th1-Biased T follicular helper (Tfh) cells in lymphoid tissues during chronic simian immunodeficiency virus infection defines functionally distinct germinal center Tfh cells. *J Immunol*. (2016) 197:1832–42. doi: 10.4049/jimmunol.1600143
97. Obeng-Adjei N, Portugal S, Tran TM, Yazew TB, Skinner J, Li S, et al. Circulating Th1-Cell-type Tfh cells that exhibit impaired B cell help are preferentially activated during acute malaria in children. *Cell Rep*. (2015) 13:425–39. doi: 10.1016/j.celrep.2015.09.004
98. Miller SA, Weinmann AS. Molecular mechanisms by which T-bet regulates T-helper cell commitment. *Immunol Rev*. (2010) 238:233–46. doi: 10.1111/j.1600-065X.2010.00952.x

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2019 Wang, Wang, Xie, Xiao, Wu, Xu, Bai, Hao, Huang, Chen, He, Li, Yang, Chen, Wu and Ye. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

Advantages of publishing in Frontiers



OPEN ACCESS

Articles are free to read
for greatest visibility
and readership



FAST PUBLICATION

Around 90 days
from submission
to decision



HIGH QUALITY PEER-REVIEW

Rigorous, collaborative,
and constructive
peer-review



TRANSPARENT PEER-REVIEW

Editors and reviewers
acknowledged by name
on published articles

Frontiers

Avenue du Tribunal-Fédéral 34
1005 Lausanne | Switzerland

Visit us: www.frontiersin.org

Contact us: info@frontiersin.org | +41 21 510 17 00



REPRODUCIBILITY OF RESEARCH

Support open data
and methods to enhance
research reproducibility



DIGITAL PUBLISHING

Articles designed
for optimal readership
across devices



FOLLOW US

[@frontiersin](https://twitter.com/frontiersin)



IMPACT METRICS

Advanced article metrics
track visibility across
digital media



EXTENSIVE PROMOTION

Marketing
and promotion
of impactful research



LOOP RESEARCH NETWORK

Our network
increases your
article's readership