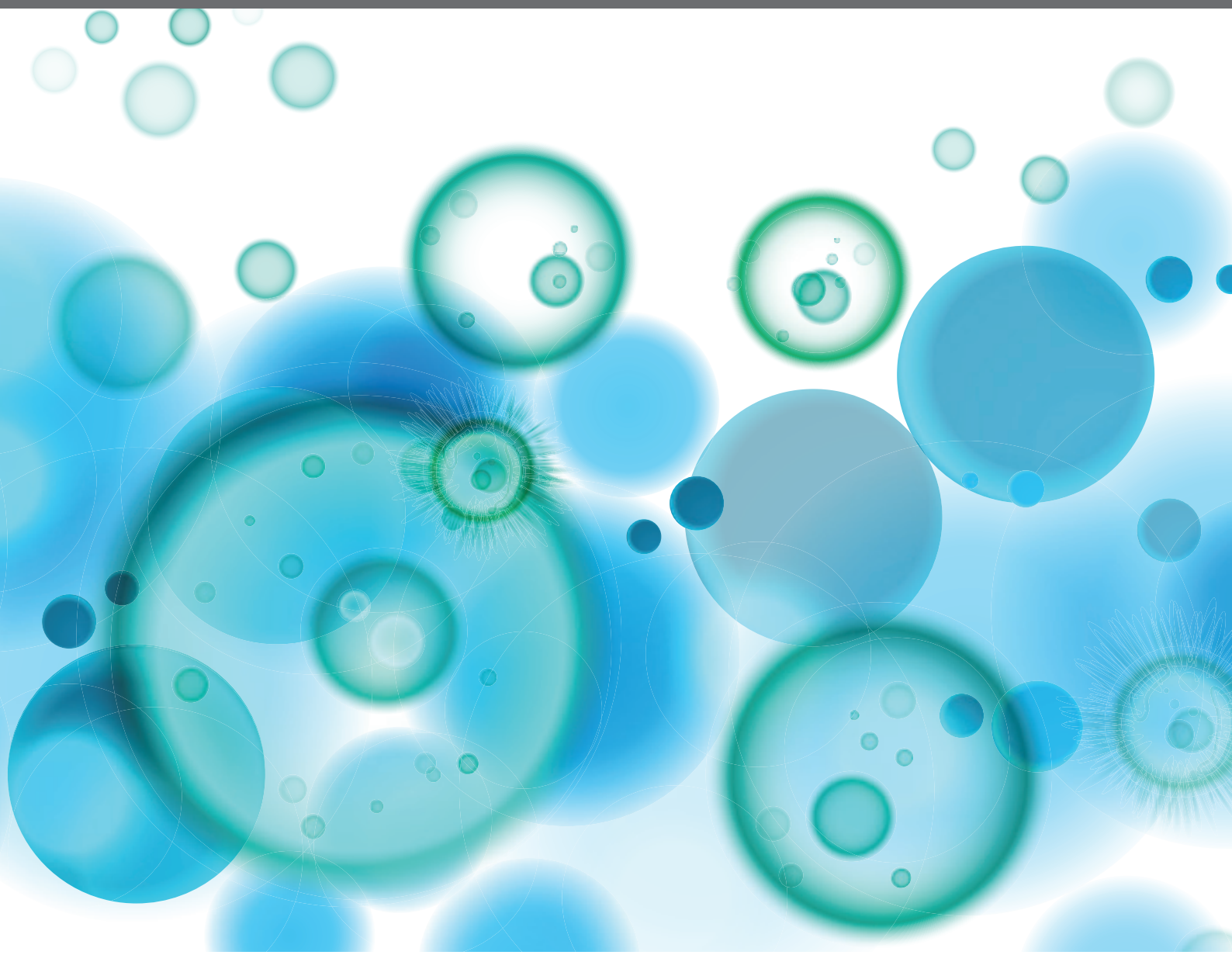
GENERATING AND SUSTAINING STABLE AUTOANTIGEN-SPECIFIC CD4 AND CD8 REGULATORY T CELLS IN LUPUS

EDITED BY: Syamal Kumar Datta, Antonio La Cava, David A. Horwitz and Ciriaco A. Piccirillo

PUBLISHED IN: Frontiers in Immunology



frontiers Research Topics

frontiers

ISSN 1664-8714
ISBN 978-2-88974-788-7
DOI 10.3389/978-2-88974-788-7

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: frontiersin.org/about/contact

GENERATING AND SUSTAINING STABLE AUTOANTIGEN-SPECIFIC CD4 AND CD8 REGULATORY T CELLS IN LUPUS

Topic Editors:
Syamal Kumar Datta, Northwestern University, United States
Antonio La Cava, University of California, Los Angeles, United States
David A. Horwitz, University of Southern California, United States
Ciriaco A. Piccirillo, McGill University, Canada

Citation: Datta, S. K., La Cava, A., Horwitz, D. A., Piccirillo, C. A., eds. (2022). Generating and Sustaining Stable Autoantigen-Specific CD4 and CD8 Regulatory T Cells in Lupus. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-88974-788-7

Table of Contents

frontiers in Immunology

EDITORIAL
published: 07 March 2022
doi: 10.3389/fimmu.2022.838604

Editorial: Generating and Sustaining Stable Autoantigen-Specific CD4 and CD8 Regulatory T Cells in Lupus

Syamal K. Datta[1]*, *David A. Horwitz*[2,3], *Ciriaco A. Piccirillo*[4,5] and *Antonio La Cava*[6]

[1] Department of Medicine, Division of Rheumatology, Northwestern University Feinberg School of Medicine, Chicago, IL, United States, [2] General Nanotherapeutics, LLC, Santa Monica, CA, United States, [3] Department of Medicine, Keck School of Medicine, University of Southern California, Los Angeles, CA, United States, [4] Department of Microbiology and Immunology, McGill University, Montreal, QC, Canada, [5] Program in Infectious Diseases and Immunology in Global Health Research Institute, McGill University, Montreal, QC, Canada, [6] Department of Medicine, University of California Los Angeles, Los Angeles, CA, United States

Keywords: autoimmunity, systemic lupus erythematosus, T regulatory cells, immune tolerance, peptide epitopes, graft-versus-host disease, nanoparticle (NP), humanized mice

Editorial on the Research Topic

Generating and Sustaining Stable Autoantigen-specific CD4 and CD8 Regulatory T Cells in Lupus

OPEN ACCESS

Edited and reviewed by:
Stephen Robert Daley, Queensland University of Technology, Australia

***Correspondence:**
Syamal K. Datta
skd257@northwestern.edu

Specialty section:
This article was submitted to Immunological Tolerance and Regulation, a section of the journal Frontiers in Immunology

Received: *18 December 2021*
Accepted: *10 February 2022*
Published: *07 March 2022*

Citation:
Datta SK, Horwitz DA, Piccirillo CA and La Cava A (2022) Editorial: Generating and Sustaining Stable Autoantigen-Specific CD4 and CD8 Regulatory T Cells in Lupus. Front. Immunol. 13:838604. doi: 10.3389/fimmu.2022.838604

CD4$^+$ AND CD8$^+$ REGULATORY T CELLS IN LUPUS

Systemic lupus erythematosus (lupus) is a complex autoimmune disease that involves major components of the immune system. It has heterogeneous clinical manifestations and, at the earliest stages, it is characterized by a deficiency of IL-2 and TGF-β, (1, 2), and epigenetic abnormalities that include an abnormal development and stability of CD4$^+$CD25$^+$CD127^{low} T regulatory (Treg) cells (3). However, merely enumerating the levels of circulating Treg cells in lupus patients has yielded inconsistent results because some of those Treg cells are functionally inactive (4, 5), and Treg cells directed to major autoantigens of lupus are not detectable in patients with active disease (6) and Robinson et al. Therefore, generating stable Treg cells that preferentially suppress pathogenic activity of self-reactive immune cells represents a critical therapeutic goal for the modulation of lupus disease, as discussed in this Research Topic.

Datta reviews the origins of the first experiments that showed that an endogenous self-antigen, namely nucleosomes from apoptotic cells, linked self-reactive lupus T helper (Th) and B cell with cognate interactions leading to the production of class-switched nephritogenic anti-dsDNA autoantibodies. Subsequently, minute doses of certain histone peptide epitopes from nucleosomes were found to induce autoantigen-specific CD4$^+$ and CD8$^+$ Treg cells. Surprisingly the epitopes were also found to render dendritic cells tolerogenic directly, which led to inhibition of multiple autoreactive cells participating in pathogenic autoimmune response in lupus.

Wei et al. review cellular mechanisms that lead to production of high-affinity autoantibodies in SLE. The onset of autoantibodies in systemic autoimmunity requires a complex and highly regulated B-T cell functional crosstalk as well as mature germinal center (GC) formation in B cell follicles of secondary lymphoid tissues. A key regulator of such events is the T follicular regulatory cell (TFR), a specialized Treg cell population that protects from hyperactivity of self-reactive T and B cells. However, recent studies show that TFRs manifest functional plasticity as they can lose Foxp3 expression and convert into disease-promoting "ex-TFRs" that acquire potent effector/

inflammatory functions. The authors review currently proposed intrinsic and extrinsic mechanisms of regulation of TFR function and discuss the roles of TFR plasticity in autoantibody production in the pathophysiology of SLE.

Singh et al. describe a Treg cell-inducing peptide called pCons that was derived from V-region sequences of anti-dsDNA autoantibodies. Singh et al. also describe the gene expression profiles and immunological markers of pCons-induced $CD8^+$ Treg cells in NZB/W lupus mice, discussing potentially interesting functional features of those pCons-induced $CD8^+$ Treg cells in the downregulation of lupus autoimmunity.

Kato and Perl critically elaborate on the recent exciting findings that showed a peripheral expansion of Treg cells in lupus patients treated with low-dose IL-2. The authors report that although IL-2 does induce $CD4^+$ Treg cells in lupus patients, in the meantime this cytokine also promotes an expansion of $CD8^+$ T cells that produce pro-inflammatory IFN-γ. This new finding raises questions on how to optimize treatments with IL-2 in lupus patients to avoid unwanted side effects while promoting the Treg cell-mediated beneficial activities on disease manifestations.

Papillion and Ballesteros-Tato point out that although IL-2 normally inhibits T follicular helper cells in SLE, IL-6 blocks this inhibitory effect, documenting that IL-6 blocked the upregulation of IL-2Rβ (CD122).

Hatzioannou et al. review the two-faced role and plasticity of Treg cells in autoimmunity and cancer, focusing on the phenotypic characteristics that Treg cells display in association with their functional flexibility. The authors elaborate on the potential therapeutic implications of targeting Treg cell plasticity either to maintain functional stability in Treg cell control of immune effector responses that sustain chronic inflammation, or to favor Treg cell destabilization in the fight against cancer.

Horwitz et al. and Robinson and Thomas review an emerging approach that avoids the use of corticosteroids and immunosuppressive drugs to treat SLE. Nanoparticles (NPs) engineered to target specific cell populations are used to repair defects in the homeostatic immune regulation, and to restore immune tolerance to autoantigens. NPs can be targeted to antigen presenting cells (APCs) to switch them from supporting pathogenic T cells to favoring the expansion of therapeutic Treg cells. Alternatively, NPs can be targeted directly to T cells for the induction and expansion of $CD4^+$ and $CD8^+$ Treg cells. Both reviews discuss potential approaches to induce autoantigen specific Treg cells, without antigen or with peptide antigens. Attention is given to lupus-specific peptide autoantigens to induce antigen-specific Treg cells and the use of NPs to function as artificial APCs (aAPCs) that induce multiple populations of lymphocytes to become regulatory cells.

Giang et al. demonstrate that aAPCs made of NPs that provide IL-2 and TGF-β to human cells can induce human $CD4^+$ and $CD8^+$ $FoxP3^+$ Treg cells both *in vitro* and *in vivo* in immunodeficient mice. These human Treg cells protect the mice from human anti-mouse graft versus host disease (GvHD). These studies suggest the possibility that repairing both the IL-2 and TGF-β defects may be necessary for a sustained disease remission in SLE.

CONCLUSIONS

This Research Topic provides updates on the influences exerted by $CD4^+$ and $CD8^+$ Treg cells on the pathogenesis and modulation of SLE disease manifestations. Among the possibilities of Treg cell modulation, epigenetic manipulations that could improve Treg cell stability and function are actively investigated and expected to be tested in clinical trials (7, 8). Several immunotherapeutic studies that focused on the numeric and functional modulation of $CD4^+$ and $CD8^+$ Treg cells, and have yielded promising laboratory results, are moving to the patient's bedside. The restoration and maintenance of Treg cell predominance over effector cells has the potential to promote long-term remission of autoimmune disease, and ultimately prevent autoimmunity in susceptible individuals.

AUTHOR CONTRIBUTIONS

All authors wrote and revised this Editorial and have approved this version for submission. SD initially conceived of, and established this Research Topic.

REFERENCES

1. Linker-Israeli M, Bakke AC, Kitridou RC, Gendler S, Gillis S, Horwitz DA. Defective Production of Interleukin 1 and Interleukin 2 in Patients With Systemic Lupus Erythematosus (SLE). *J Immunol (Baltimore Md: 1950)* (1983) 130(6):2651–5.
2. Ohtsuka K, Gray JD, Stimmler MM, Toro B, Horwitz DA. Decreased Production of TGF-Beta by Lymphocytes From Patients With Systemic Lupus Erythematosus. *J Immunol* (1998) 160:2539–45.
3. Sakaguchi S. Taking Regulatory T Cells Into Medicine. *J Exp Med* (2021) 218 (6):e20210831. doi: 10.1084/jem.20210831
4. Valencia X, Yarboro C, Illei G, Lipsky PE. Deficient CD4+CD25high T Regulatory Cell Function in Patients With Active Systemic Lupus Erythematosus. *J Immunol* (2007) 178(4):2579–88. doi: 10.4049/jimmunol.178.4.2579
5. Miao J, Zhu P. Functional Defects of Treg Cells: New Targets in Rheumatic Diseases, Including Ankylosing Spondylitis. *Curr Rheumatol Rep* (2018) 20 (5):30. doi: 10.1007/s11926-018-0729-1
6. Zhang L, Bertucci AM, Ramsey-Goldman R, Burt RK, Datta SK. Regulatory T Cell (Treg) Subsets Return in Patients With Refractory Lupus Following Stem Cell Transplantation, and TGF-β-Producing CD8 + Treg Cells Are Associated With Immunological Remission of Lupus. *J Immunol* (2009) 183(10):6346–58. doi: 10.4049/jimmunol.0901773
7. Kawakami R, Kitagawa Y, Chen KY, Arai M, Ohara D, Nakamura Y, et al. Distinct Foxp3 Enhancer Elements Coordinate Development, Maintenance, and Function of Regulatory T Cells. *Immunity* (2021) 54(5):947–61.e8. doi: 10.1016/j.immuni.2021.04.005
8. Dikiy S, Li J, Bai L, Jiang M, Janke L, Zong X, et al. A Distal Foxp3 Enhancer Enables Interleukin-2 Dependent Thymic Treg Cell Lineage Commitment for

Robust Immune Tolerance. *Immunity* (2021) 54(5):931–46.e11. doi: 10.1016/j.immuni.2021.03.020

Conflict of Interest: DAH is the CEO of and holds stock in General Nanotherapeutics, LLC.

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.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Immunology

ORIGINAL RESEARCH
published: 02 March 2021
doi: 10.3389/fimmu.2021.635531

Double-Edged Sword: Interleukin-2 Promotes T Regulatory Cell Differentiation but Also Expands Interleukin-13- and Interferon-γ-Producing CD8$^+$ T Cells *via* STAT6-GATA-3 Axis in Systemic Lupus Erythematosus

Hiroshi Kato and Andras Perl *

Division of Rheumatology, Departments of Medicine, Microbiology and Immunology, and Biochemistry and Molecular Biology, College of Medicine, State University of New York, Upstate Medical University, Syracuse, NY, United States

OPEN ACCESS

Edited by:
Antonio La Cava, University of California, Los Angeles, United States

Reviewed by:
José Carlos Crispín, Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán (INCMNSZ), Mexico
Sokratis A. Apostolidis, Hospital of the University of Pennsylvania, United States

***Correspondence:**
Andras Perl
perla@upstate.edu

Specialty section:
This article was submitted to Immunological Tolerance and Regulation, a section of the journal Frontiers in Immunology

Received: *30 November 2020*
Accepted: *13 January 2021*
Published: *02 March 2021*

Citation:
Kato H and Perl A (2021) Double-Edged Sword: Interleukin-2 Promotes T Regulatory Cell Differentiation but Also Expands Interleukin-13- and Interferon-γ-Producing CD8$^+$ T Cells via STAT6-GATA-3 Axis in Systemic Lupus Erythematosus. Front. Immunol. 12:635531. doi: 10.3389/fimmu.2021.635531

Interleukin-2 (IL-2) expands the depleted T regulatory (Treg) cell population, and it has emerged as a potential therapy in systemic lupus erythematosus (SLE). However, IL-2 administration may involve the risk of expanding unwanted pro-inflammatory cells. We herein studied the effects of IL-2 on pro-inflammatory cytokine production by CD4$^+$ and CD8$^+$ T cells in parallel with Treg development following CD3/CD28 co-stimulation. While Treg cells are depleted in SLE patients, their CD4$^+$ T cells were poised to receive and activate IL-2 signaling as evidenced by upregulation of CD25 and enhanced IL-2-incued STAT5 phosphorylation during Treg differentiation. In patients with SLE, however, IL-2 also expanded CD8$^+$ T cells capable of producing interleukin-5, interkeukin-13 (IL-13), and interferon-γ (IFN-γ) that occurred with enhanced expression of GATA-3 and phosphorylation of STAT6 but not STAT5. Our data pinpoint a safety signal for systemic administration of IL-2 and challenges a long-held conceptual platform of type 1 and 2 cytokine antagonism by newly documenting the IL-2-dependent development of IL-13 and IFN-γ double-positive (IL-13$^+$IFNγ$^+$) CD8$^+$ T cells in SLE.

Keywords: systemic lupus erythematosus, interleukin-2 (IL-2), interleukin-13 (IL-13), interferon-γ (IFN-γ), T regulatory (Treg) cell, CD8$^+$ T cells, STAT6, GATA-3

INTRODUCTION

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by aberrant T- and B-cell activation culminating in a production of antinuclear antibodies (1). Among numerous immune dysregulation pathways implicated in the pathogenesis, the depletion of T regulatory (Treg) cells has emerged as an important mediator of the disease (2–4). In this respect, interleukin (IL)-2 elicits T regulatory (Treg) cell differentiation in association with phosphorylation of STAT5 and its binding

to the *Foxp3* gene (5). These findings along with IL-2 deficiency in lupus patients (6) yielded the notion that supplementation of IL-2 could restore the immune tolerance by expanding the Treg cell population. Indeed, low dose IL-2 therapy was shown to expand Treg cells and ameliorate the lupus disease activity (7).

Non-selective administration of IL-2, however, poses a concern for potential expansion of unwanted pro-inflammatory cells in view of its pleiotropic functions, in particular, its role as a T-cell growth factor (8). In fact, IL-2 induces Th1 and Th2 differentiation in a STAT5-dependent manner (9, 10). In addition, IL-2 elicits the differentiation of naïve $CD8^+$ T cells into effector and memory cytotoxic T cells along with the induction of interferon (IFN)-γ, perforin, and granzymes (11).

While extensive evidence underpins the essentiality of $CD4^+$ and $CD4^-CD8^-$ double-negative T cells in lupus pathogenesis (12–14), roles for $CD8^+$ T cells have also been increasingly recognized. $CD8^+$ T cells during a lupus flare exhibit more prominent cytotoxic phenotype and functions than during remission, and the frequency of such cells correlates with the SLE disease activity index (SLEDAI) score (15). Tubulointerstitial nephritis associated with $CD8^+$ T cell infiltrates confers an increased risk for progressive lupus nephritis (16). With regard to the type of immune response mediated by T cells, it remains controversial whether SLE is driven by type 1 or type 2 immunity given the various animal models showing discrepant findings. In humans, some studies showed increased IL-4, but decreased IFN-γ in lupus patients (17), whereas others indicate the importance of IFN-γ in diffuse proliferative lupus nephritis (18). SLE patients with higher SLEDAI score have lower IFN-γ but higher IL-4 expression than those with lower SLEDAI score (19). Another type 2 cytokine, IL-13, shares many biological functions with IL-4, as exemplified by when human IL-13 elicits B-cell proliferation and its immunoglobulin production (20–22). In addition to the contribution of IL-13 to asthma and allergic disorders (23, 24), it is important to note that GATA-3-dependent IL-13 production by $CD8^+$ T cells promotes fibrosis in systemic sclerosis (25, 26). While our understanding of lupus T cell biology has been rapidly evolving, it is unknown how IL-2 affects the lineage-specification of lupus T cells, and whether IL-13 plays a role in immune dysregulation in SLE.

In this study, we evaluated the effects of IL-2 on pro-inflammatory cytokine expression in SLE $CD4^+$ and $CD8^+$ T cells in comparison to those on Treg cell development. While Treg population is depleted in SLE patients, their $CD4^+$ T cells were primed to receive and activate IL-2 signaling as evidenced by upregulation of CD25 and enhanced IL-2-induced STAT5 phosphorylation during Treg differentiation. On the other hand, SLE $CD8^+$ T cells produced greater amount of IL-13 and IFN-γ than $CD4^+$ T cells in an IL-2-dependent manner. In addition, IL-2 expanded the IL-13-producing lupus $CD8^+$ T cells that also expressed IL-5 and IFN-γ in association with STAT6 phosphorylation and GATA-3 expression, but not with STAT5 phosphorylation. Our data conveys a clear safety signal in further pursuit of systemic administration of IL-2, and supports the rationale for Treg-cell-targeted delivery of IL-2 in the treatment of SLE.

MATERIALS AND METHOD

Human Subjects

In total, 33 patients with SLE fulfilling the American College of Rheumatology diagnostic criteria (27) were studied. In each experiment, peripheral blood was obtained from SLE patients (all female) and healthy control (HC) subjects who were matched to the patients by age (within 10 years), sex, and ethnic background. Age of study participants was 44.8 ± 2.0 (mean ± SD) years in SLE, and 44.2 ± 1.9 years in HC subjects. Disease activity was assessed by the SLEDAI scores (28), which ranged from 0 to 34 (mean ± SD: 6.24 ± 1.17). Mean daily prednisone dose was 6.52 ± 1.62 mg. Immunosuppressive drugs taken by the study subjects included hydroxychloroquine (N=31), methotrexate (N=1), mycophenolate mofetil (N=10), mycophenolic acid (N=1), azathioprine (N=5), cyclosporine (N=1), tacrolimus (N=1), belimumab (N=6), and abatacept (N=1). The study was approved by the Institutional Review Board at the SUNY Upstate Medical University.

Isolation of Untouched T Cells and Cell Culture

Peripheral blood mononuclear cells (PBMCs) were isolated by using Ficoll Histopaque gradient (GE Health Care Bio-Sciences). $CD3^+$ T cells were isolated by negative selection using untouched human T cell isolation kit (Life technologies, Cat# 11344D). Purity of $CD3^+$ T cells was confirmed to be above 97%. Cells were cultured in RPMI culture media with 10% FCS, 1% Penicillin/Streptomycin, and 1% L-glutamine (all from Corning CellGro except for FCS, which was from Gibco) for 3 days, and stained with PE Cy7-conjugated anti-CD4 (Clone: SK3, Cat# 557852, RRID : AB_396897) and phycoerythrine (PE)-conjugated anti-CD25 (Clone: M-A251, Cat# 555432, RRID : AB_395826, both from BD Biosciences). The cells were permeabilized as per the manufacturer's instructions and stained with AF-647-conjugated anti-FoxP3 (Biolegend, Clone: 150D, Cat# 320014, RRID : AB_439750).

Isolation of $CD4^+$ and $CD8^+$ T Cells and Cell Culture

$CD4^+$ and $CD8^+$ T cells were isolated by negative selection using human $CD4^+$ T cell enrichment (Cat# 15062) and $CD8^+$ T cell isolation kits (Cat# 17953, both from STEMCELL), respectively. Cells were cultured for 3 days in the presence of plate-bound anti-CD3 (anti TCR ϵ hybridoma from ATCC) and soluble anti-CD28 (1 μg/ml, BD Biosciences, Clone: CD28.2, Cat# 555725, RRID : AB_396068) in the presence or absence of IL-2 (100 IU/ml, Peprotech, Cat# 200-02) or anti-IL-2 (100 or 1000 ng/ml, R&D Systems, Cat# MAB202, RRID : AB_2264789).

Intracellular Staining

Cells were pre-incubated with PMA (5 ng/ml) and ionomycin (500 ng/ml) for 6 h, and with brefeldin A for 5 h (10 μg/ml; all

from Sigma-Aldrich). For cytokine detection, FITC-conjugated anti–IFN-γ (Clone: B27, Cat# 554700, RRID : AB_395517), PE-conjugated anti-IL-5 (Clone: JES1-39D10, Cat# 559332, RRID : AB_397229), or IL-17 (Clone: SCPL1362, Cat# 560436, RRID : AB_1645514), BV711 conjugated anti-IL-13 (Clone: JES10-5A2, Cat# 564288, RRID : AB_2738731), and allophycocyanin-conjugated anti-IL-4 (Clone: 8D4-8, Cat# 560671, RRID : AB_1727546) or IL-21 (Clone: 3A3-N2.1, Cat# 562043, RRID : AB_10896655, all from BD Biosciences) were used alone or together. Isotype control Abs included FITC-conjugated mouse IgG1 κ (Clone: MOPC-21, Cat# 551954, RRID : AB_394297), PE-conjugated rat IgG2a κ (Clone: R35-95, Cat# 559317, RRID : AB_10050484), BV711-conjugated rat IgG1 κ (Clone: R3-34, Cat# 563283, RRID : AB_2869482), and Alexa Fluor (AF)-647-conjugated mouse IgG1 κ (Clone: MOPC-21, Cat# 557732, RRID : AB_396840, all from BD Biosciences).

T Regulatory Cell Polarization

Naive $CD4^+$ T cells were isolated from SLE and matched HC subjects by using Human Naive $CD4^+$ T cell Enrichment Kit (STEMCELL, Cat# 19555). The purity of naive $CD4^+$ T cells as defined by the proportion of $CD4^+CD45RA^+CD62L^+$ cells was above 99%. Cells were cultured for 72 h in the presence of anti-CD3/CD28 and TGF-β (5 ng/ml, Peprotech, Cat# 100-21) with IL-2 (50 IU/ml) or anti-IL-2 (100 or 1000 ng/ml). Cells were stained with FITC-conjugated anti-CD25 (Clone: M-A251, Cat# 555431, RRID : AB_395825) and AF-647-conjugated anti-FoxP3 (Clone: 259D/C7, Cat# 560045, RRID : AB_1645411 both from BD Biosciences). Frequency of $CD4^+CD25^+FOXP3^+$ cells was determined by flow cytometry. In other experiments, $CD4^+$ T cells isolated from matched SLE and HC subjects were cultured for 3 days in the presence of anti-CD3/CD28 and TGF-β (20 ng/ml) with or without IL-2 (100 IU/ml) or anti-IL-2 (100 ng/ml).

Immunoblotting

Using lysates of $CD4^+$ T cells cultured under Treg-polarizing conditions, total STAT5 (Clone: A-9, Cat# sc-74442, RRID : AB_1129711) and its phosphorylation at tyrosine 694 ($pSTAT5^{Y694}$, Clone: C11C5, Cat# 9359, RRID : AB_823649) were detected by immunoblotting. Using lysates of $CD8^+$ T cells cultured in the presence or absence of IL-2 or anti-IL-2, phosphorylation of STAT5 at tyrosine 694, phosphorylation of STAT6 at tyrosine 641 ($pSTAT6^{Y641}$, Clone: C11A12, Cat# 9364, RRID : AB_2271227), and expression of GATA-3 (Clone: HG3-31, Cat# sc-268, RRID : AB_2108591) were determined by immunoblotting (anti-STAT5 and anti-GATA-3 were from Santa Cruz Biotechnology whereas the remainder was from Cell Signaling Technology). The signal intensity was normalized to that of actin (Millipore, Clone: C4, Cat# MAB1501, RRID : AB_2223041).

Statistical Analysis

Student t test was performed for comparison of phenotype between two groups with two-tailed p values < 0.05 considered significant. Two-way ANOVA was followed by Bonferroni's posttest for multiple comparisons using Prism 8 software (GraphPad, La Jolla, CA). Association of two variables was determined by Pearson's and Spearman's correlation analyses.

RESULTS

Systemic Lupus Erythematosus CD4+ T Cells Are Poised to Receive Interleukin-2 Signaling During T Regulatory Cell Differentiation

To confirm the essentiality of IL-2 signaling in Treg differentiation in SLE, naïve $CD4^+$ T cells were cultured under Treg-polarizing conditions in the presence or absence of IL-2 or anti-IL-2. IL-2 blockade abrogated Treg differentiation both in SLE and healthy control subjects (**Figure 1A**). Of note, supplementation of IL-2 induced STAT5 phosphorylation in SLE $CD4^+$ T cells, but not in healthy control $CD4^+$ cells (**Figure 1B**). Such a heightened sensitivity to IL-2 prompted us to examine the expression of IL-2 receptor α chain (CD25) on SLE $CD4^+$ T cells (29). Instead of immunophenotyping cells immediately after isolation, untouched $CD3^+$ T cells were rested in culture media without anti-CD3/CD28 stimulation for 3 days to eliminate the effects of immunosuppressive drugs that the study subjects had received. Although $CD4^+CD25^+FOXP3^+$ Treg population was depleted, CD25 was upregulated on lupus $CD4^+$ T cells, resulting in diminished proportion of $CD4^+CD25^+FOXP3^+$ Treg cells among the $CD4^+CD25^+$ cells in SLE (**Figure 1C**). Collectively, although SLE $CD4^+$ T cells are primed to receive and activate IL-2 signaling as evidenced by the upregulation of CD25 and increased IL-2-induced STAT5 phosphorylation under Treg-polarizing conditions, Treg cells are depleted in SLE patients (**Figure 1C**). However, it is corrected once cells are stimulated with anti-CD3/CD28 (**Figure 1A** (12, 30),) likely because of a large amount of IL-2 produced by T cells (30). These findings point to the IL-2 deficiency underlying the SLE Treg depletion (6).

Interleukin-2 Elicits the Expansion of Interleukin-13-Producing $CD8^+$ T Cells That Also Express Interleukin-5 and Interferon-γ

To evaluate the roles for IL-2 in pro-inflammatory cytokine expression in SLE T cells, magnetically isolated $CD4^+$ and $CD8^+$ T cells were cultured in the presence or absence of IL-2 or anti-IL-2. IL-2 positively controls the expression of IL-5, IL-13, and IFN-γ, but not IL-4, in particular, in $CD8^+$ T cells (**Figures 2**, **3**, **S1**). $CD4^+$ T cells appeared to be the predominant source of IL-21, and it was not IL-2 dependent (**Figure S2**). No meaningful IL-17 expression was observed (data not shown). Among these IL-2-dependennt cytokines, $CD8^+$ T cells produced greater amount of IL-13 (HC: 30.41 ± 2.36%, 1.51 ± 0.14%, $p<0.0001$; SLE: 37.91 ± 2.27%, 1.53 ± 0.22%, $p<0.0001$) and IFN-γ (HC: 46.00 ± 3.22%, 25.07 ± 6.89%, p=ns; SLE: 48.04 ± 4.46%, 29.53 ± 2.83%, $p<0.01$) than $CD4^+$ T cells (**Figures 2** and **3**). IL-13 expression in $CD8^+$ T cells was greater than that in $CD4^+$ T cells on a per cell basis as determined by the mean fluorescence intensity (MFI) (HC: 396.67 ± 26.54, 165.64 ± 6.10, $p<0.0001$; SLE: 470.42 ± 27.39, 158 ± 8.65, $p<0.0001$). Of note, SLE $CD8^+$ T cells produced greater amount of IL-13 (% positive cells: SLE: 37.91 ± 2.27%, HC: 30.41 ± 2.36%, $p<0.05$; MFI: SLE: 470.42 ±

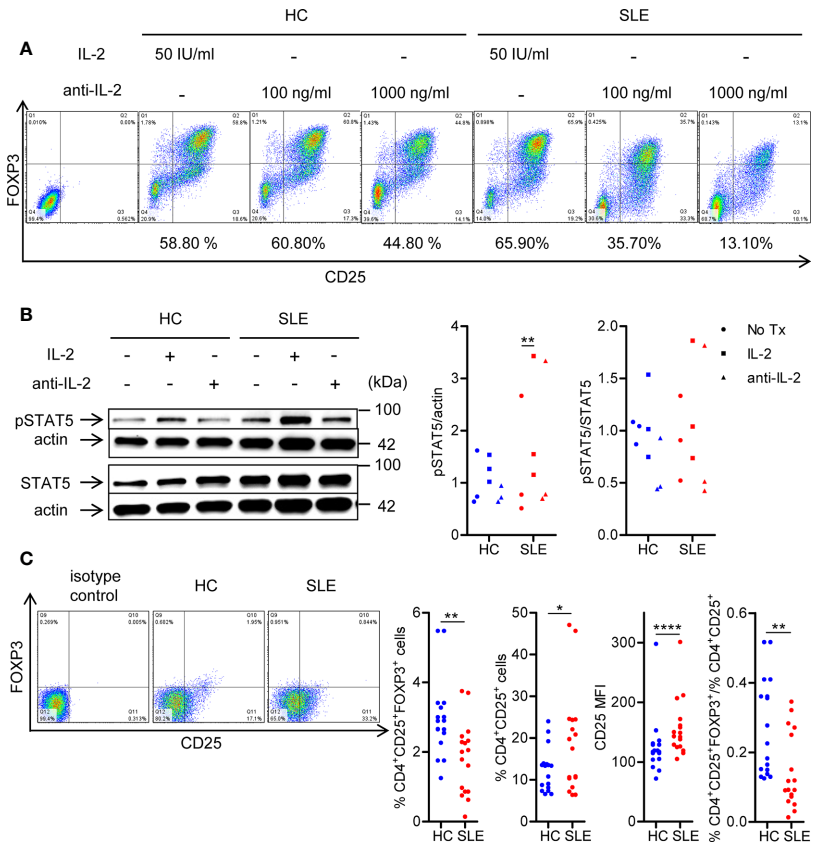


FIGURE 1 | SLE $CD4^+$ T cells are poised to activate IL-2 signaling during Treg differentiation. **(A)** Naïve $CD4^+$ T cells were isolated from a systemic lupus erythematosus (SLE) patient and matched healthy control (HC) subject, and cultured for 3 days in the presence of anti-CD3/CD28 and TGF-β (5 ng/ml) with IL-2 (50 IU/ml) or anti-IL-2 (100 or 1,000 ng/ml). The frequency of $CD4^+CD25^+FOXP3^+$ cells was determined by flow cytometry. Numbers below the plots represent the frequency of $CD4^+CD25^+FOXP3^+$ Tregs. The dot plots on the left end represent isotype control staining. **(B)** $CD4^+$ T cells isolated from matched SLE and HC subjects were cultured for 3 days in the presence of anti-CD3/CD28 and TGF-β (20 ng/ml) with or without IL-2 (100 IU/ml) or anti-IL-2 (100 ng/ml). Total STAT5 and its phosphorylation at tyrosine 694 were detected by immunoblotting. Representative immunoblot staining (left panel). The signal intensity of phospho-STAT5 and total STAT5 was normalized to that of actin. The normalized pSTAT5 signal intensity (middle panel) and the ratio of normalized pSTAT5 signal intensity over normalized STAT5 signal intensity (right panel) from 3 pairs of matched HC and SLE subjects. **(C)** Untouched T cells from matched SLE and HC subjects were cultured for 3 days without anti-CD3/CD28 stimulation. Expression of CD25 and FOXP3 in $CD4^+$ cells were determined by flow cytometry. Representative flow cytometry dot plots are shown (left panel). Cumulative data of frequency of $CD4^+CD25^+FOXP3^+$ and $CD4^+CD25^+$ cells, mean fluorescence intensity (MFI) of CD25 expression in $CD4^+$ T cells, and the proportion of $CD4^+CD25^+FOXP3^+$ cells among $CD4^+CD25^+$ cells from 17 pairs of matched SLE and HC subjects (right panel). Data were analyzed by a paired two-tailed t-test (*p<0.05, **p<0.01, ****p<0.0001).

27.39, HC: 396.67 ± 26.54, p<0.05) than healthy control $CD8^+$ T cells (**Figures 2** and **3A**).

We next turned to the correlation analysis of type 1 and 2 cytokine expression. IL-13 expression was greater in IL-5-expressing cells than IL-5-non-expressing cells both in $CD4^+$ and $CD8^+$ T cells (**Figure S3A**). Accordingly, the expression of IL-13 and IL-5 was positively correlated in $CD8^+$ T cells (**Figure S3B**), yielding a $IL\text{-}13^+$ $IL\text{-}5^+$ $CD8^+$ T cell population that contracted in association with IL-2 blockade (**Figure 2**). In contrast, the expression of IL-13 and IL-4 appeared to be mutually exclusive (**Figure S1A**), and there was no difference of IL-13 expression between IL-4-expressing and -non-expressing cells (**Figure S3A**). Unexpectedly, IL-13 expression in IFN-γ-expressing $CD8^+$ T cells was greater than that in IFN-γ-non-expressing $CD8^+$ T cells (**Figure S3A**). Along this line, there was a positive correlation between the expression of IL-13 and IFN-γ (**Figure S3B**), resulting in the expansion of $IL\text{-}13^+$ $IFN\text{-}\gamma^+$ double-positive $CD8^+$ T cells in SLE (SLE: 20.53 ± 1.48%, HC: 16.15 ± 1.48%, p<0.05, **Figures 3A, B**) which contracted in association with IL-2 blockade. Collectively, IL-2 expanded IL-13-expressing $CD8^+$ T cells including an $IL\text{-}13^+$ $IFN\text{-}\gamma^+$ double-positive subset in SLE.

Interleukin-13 and Interleukin-5 Expression Are Associated with Interleukin-2-Induced STAT6 Phosphorylation and GATA-3 Expression in $CD8^+$ T Cells

To understand the mechanisms by which IL-2 induces the expansion of IL-13- and IL-5-producing $CD8^+$ T cells,

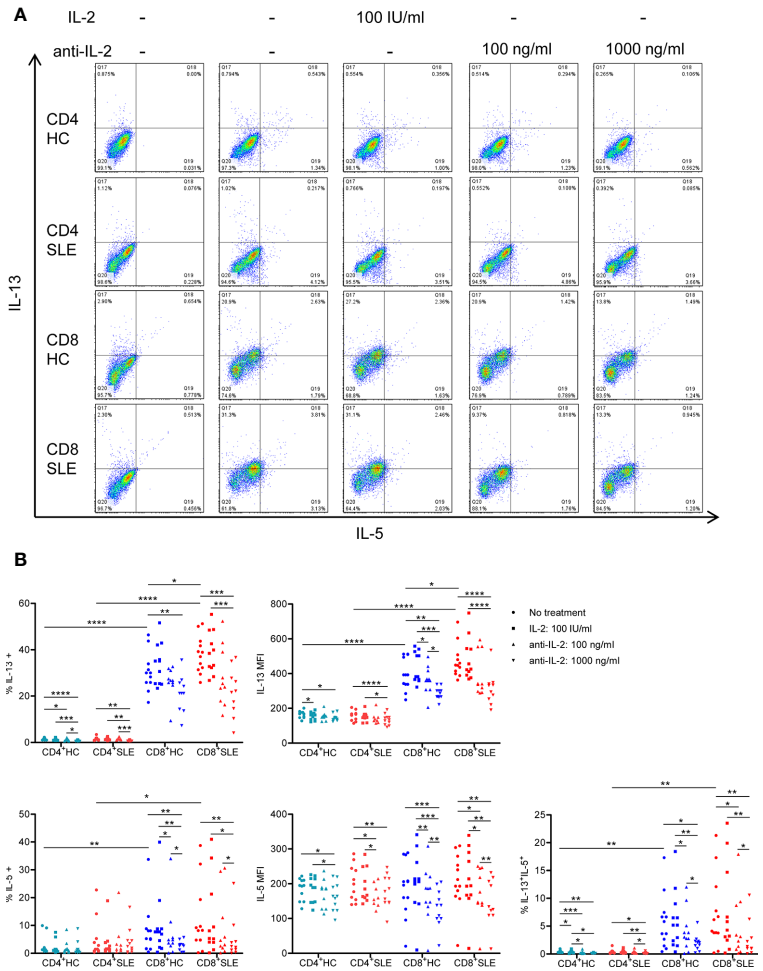


FIGURE 2 | Systemic lupus erythematosus (SLE) $CD8^+$ T cells produce increased IL-13 in an IL-2-dependent manner. **(A)** $CD4^+$ and $CD8^+$ T cells isolated from matched SLE and health control (HC) subjects were cultured for 3 days with anti-CD3/CD28 in the presence or absence of IL-2 (100 IU/ml) or anti-IL-2 (100 or 1,000 ng/ml). IL-5 and IL-13 expression was determined by flow cytometry. Representative flow cytometry dot plots are shown. The dot plots on the left end represent isotype control staining. **(B)** Cumulative data of MFI and the frequency of expression of individual cytokines from 12 pairs of matched SLE and HC subjects. Statistical analysis was made by two-way ANOVA followed by Bonferroni's correction for multiple comparisons (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

phosphorylation of STAT5 and STAT6 and GATA-3 expression were determined in the lysates of $CD8^+$ T cells cultured in the presence or absence of IL-2 or anti-IL-2. Neutralization of IL-2 profoundly diminished the phosphorylation of STAT6 and GATA-3 expression in SLE $CD8^+$ T cells; however, with regard to the impact of IL-2 blockade on STAT5 phosphorylation, such dose-dependent abolishment of phospho-STAT5 was not observed (**Figures 4A, B**). Of note, the expression of IL-13 and IL-5 were positively correlated with STAT6 phosphorylation and GATA-3 expression, but negatively correlated with STAT5 phosphorylation in $CD8^+$ T cells (**Figure 4C**). In this respect, we previously documented increased GATA-3 expression in SLE $CD8^+$ T cells as compared with healthy control $CD8^+$ T cells (12). In contrast to these type 2 cytokines, IL-4 expression was not correlated with STAT5 or STAT6 phosphorylation or GATA-3 expression (data not shown). Unexpectedly, there was a positive correlation between the expression of IFN-γ and GATA-3 in $CD8^+$ T cells (**Figure 4C**), which may account for the concurrent expression of IL-13 and IFN-γ (**Figure 3**). Our data collectively suggests that IL-2 induces IL-13, IL-5, and IFN-γ expression in lupus $CD8^+$ T cells *via* STAT6-GATA-3 dependent mechanisms in contrast to the Treg differentiation in which IL-2-STAT5 axis plays a more essential role (**Figure 1**).

DISCUSSION

In this study, we documented that IL-2-STAT5 pathway was more critical to Treg differentiation in SLE than in health control subjects. On the other hand, IL-2 expanded IL-13-producing $CD8^+$ T cells that also expressed IL-5 and IFN-γ in lupus patients *via* a signaling pathway likely involving the activation of STAT6-

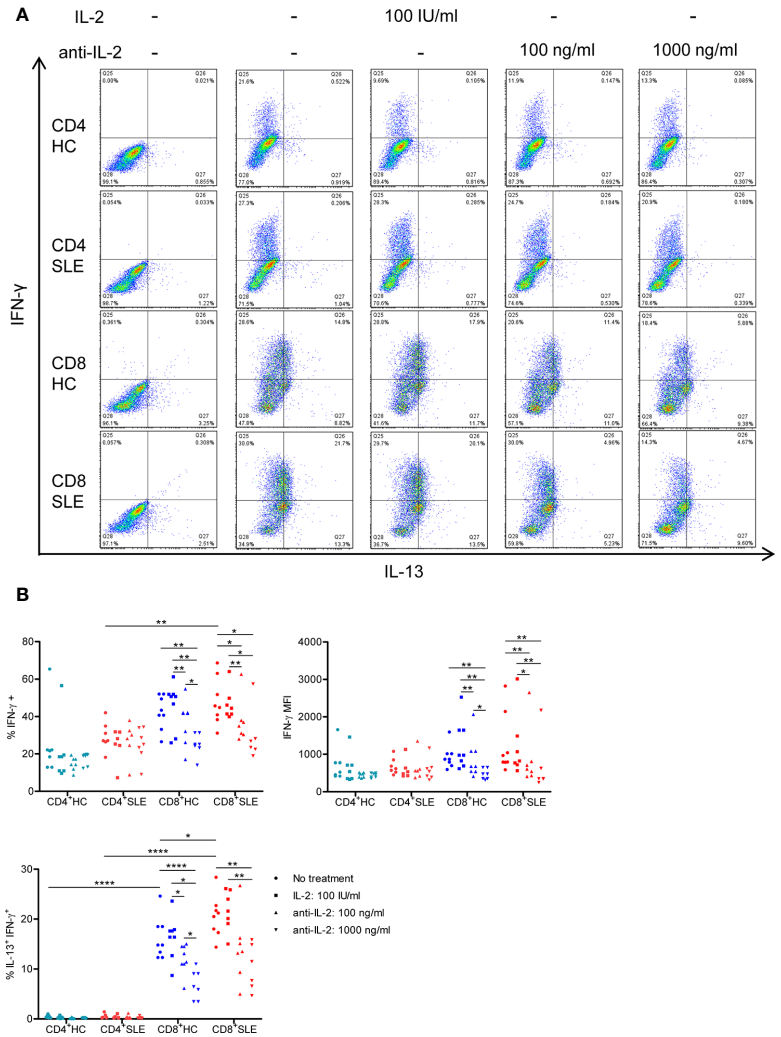


FIGURE 3 | IL-2 expands IL-13$^+$IFN-γ$^+$ CD8$^+$ T cells in systemic lupus erythematosus (SLE). **(A)** CD4$^+$ and CD8$^+$ T cells from matched SLE and health control (HC) subjects were cultured as described in **Figure 2**, and IL-13 and IFN-γ expression was determined by flow cytometry. **(B)** Cumulative data of mean fluorescence intensity (MFI) of IFN-γ and the frequency of IFN-γ$^+$ and IL-13$^+$IFN-γ$^+$ cells. Statistical analysis was made by two-way ANOVA followed by Bonferroni's correction for multiple comparisons (*p<0.05, **p<0.01, ****p<0.0001).

GATA-3 axis, but not STAT5. It is important to note that lupus CD4$^+$ T cells were primed to receive and activate IL-2 signaling as evidenced by the upregulation of CD25 and enhanced IL-2-induced STAT5 phosphorylation during Treg differentiation even though the CD4$^+$CD25$^+$FOXP3$^+$ Treg population was depleted in SLE patients. However, the Treg depletion is corrected by anti-CD3/CD28 stimulation *in vitro* (12, 30). A series of these findings pinpoint the IL-2 deficiency underlying the Treg depletion in SLE (6).

While it is not clear how IL-2 activates STAT6 and GATA-3 in CD8$^+$ T cells, our data provides compelling evidence that this pathway is STAT5 independent. The neutralization of IL-2 did not have a robust impact on STAT5 phosphorylation unlike STAT6 phosphorylation and GATA-3 expression. In addition, the expression of IL-13 and IL-5 were negatively correlated with STAT5 phosphorylation in CD8$^+$ T cells. It is worth noting here that IL-2-dependent STAT5 phosphorylation and GATA-3 expression are essential for IL-4-independent early IL-4 expression in CD4$^+$ T cells (31). These observations suggest that IL-2 utilizes different signaling pathways in eliciting the activation and differentiation of CD4$^+$ Treg cells, CD4$^+$ non-Treg cells, and CD8$^+$ T cells. Unlike the neutralization of IL-2, the supplementation of IL-2 had no effects on the expression of IL-5 and IL-13 and IFN-γ, which is attributed to a large amount of IL-2 produced by T cells in response to CD3/CD28 stimulation alone (30).

Although IL-2 activates the mechanistic target of rapamycin (mTOR), its blockade by sirolimus elicits Treg expansion both *in vitro* (30) and *in vivo* (32). This suggests that mTOR activation is not essential for IL-2-induced Treg expansion. IL-2 also activates the Akt-mTOR axis in CD8$^+$ T cells (33). Our previous study showed that mTOR blockade restrained GATA-3 expression in

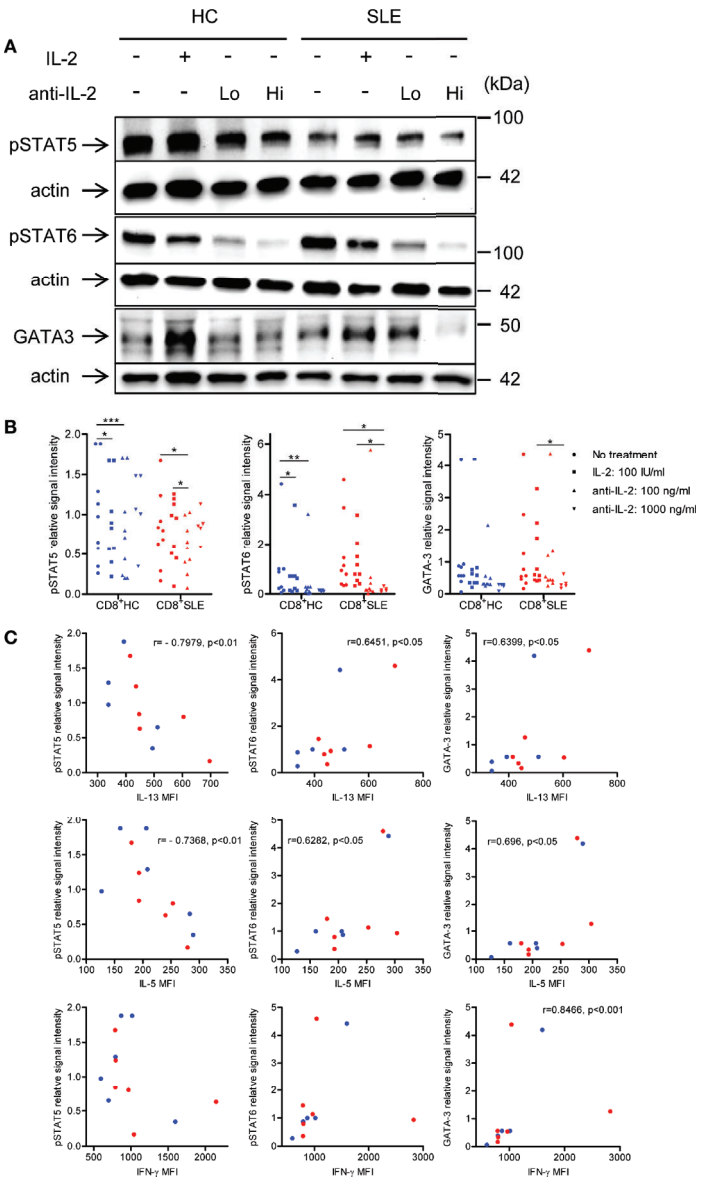


FIGURE 4 | IL-2 induces STAT6 phosphorylation and GATA3 expression in systemic lupus erythematosus (SLE) $CD8^+$ T cells. **(A)** $CD8^+$ T cells from matched SLE and health control (HC) subjects were cultured as described in **Figure 2**. Expression of GATA-3 and phosphorylation of STAT5 at tyrosine 694 and STAT6 at tyrosine 641 were detected by immunoblotting. Representative immunoblot staining was presented. Lo and Hi concentrations of anti-IL-2 denote 100 and 1,000 ng/ml, respectively. **(B)** The signal intensity of phospho-STAT5, phospho-STAT6, and GATA-3 were normalized to that of actin. Cumulative data from 9 pairs of matched HC and SLE subjects. Data were analyzed by a two-tailed t-test (*p<0.05, **p<0.01, ***p<0.001). **(C)** Pearson's and Spearman's correlation analyses were performed to determine the association between the expression of cytokines (IL-13, IL-5, and IFN-γ) and transcription factors (phospho-STAT5, phospho-STAT6, and GATA-3). The blue and red plots represent data from HC and SLE patients, respectively. Spearman correlation coefficient was presented for the association between IL-13 and phospho-STAT5, IL-5 and phospho-STAT-5, and IFN-γ and GATA-3. Pearson correlation coefficient was presented for the remainder of associations.

$FOXP3^-CD4^+$ T cells (30). These findings suggest that the IL-2-Akt-mTOR axis may also affect GATA-3 expression in $CD8^+$ T cells.

Among the three type 2 cytokines studied, there was a positive correlation between the expression of IL-13 and IL-5. IL-2 blockade abolished the expression of IL-13 and IL-5 in association with diminished STAT6 phosphorylation and GATA-3 expression. Additionally, the expression of these type 2 cytokines was positively correlated with STAT6 phosphorylation and GATA-3 expression. We previously reported increased GATA-3 expression in lupus $CD8^+$ T cells (12). A series of these findings suggest that STAT6-GATA-3 axis drives the expression of IL-13 and IL-5 in $CD8^+$ T cells. Conversely, the expression of IL-13 and IL-4 appeared mutually exclusive, and there was no

appreciable size of IL-13$^+$ IL-4$^+$ population. Furthermore, IL-4 expression was resistant to IL-2-blockade-mediated abrogation of STAT6 phosphorylation and GATA-3 expression, and not correlated with these transcription factors. While our data does not exclude the involvement of STAT6-GATA-3 axis in the initial commitment to IL-4-expressing lineage as the study was not conducted on naïve CD8$^+$ T cells, it suggests that CD8$^+$ T cells maintain their IL-4 expression by STAT6-GATA-3-independent mechanisms.

While concurrent IFN-γ and IL-4 expression in CD4$^+$ T cells has been reported (34), this is the first study documenting a dual expression of IL-13- and IFN-γ in CD8$^+$ T cells. GATA-3 blocks IFN-γ expression (35, 36), whereas T-bet suppresses IL-4 and IL-5 expression in CD4$^+$ T cells (37). Nonetheless, as such an antagonism has not been documented in CD8$^+$ T cells, it is plausible that lupus CD8$^+$ T cells employ a distinct molecular mechanism that allows for a dual IL-13- and IFN-γ expression. We identified a positive correlation between the expression of IFN-γ and GATA-3 in CD8$^+$ T cells. Instead of proposing GATA-3 as a driver of IFN-γ expression in CD8$^+$ T cells, we reason that IL-2 signaling activates a transcription factor upstream of both type 1 and type 2 programs. One such candidate is Notch as it was shown to concurrently regulate both Th1 and Th2 programs (38). Whether these cells in lupus patients are equipped with both helper- and cytotoxic functions would merit further investigation.

In conclusion, our data suggest that IL-2 supplementation may be a double-edged sword in the treatment of SLE, and supports the importance of Treg-cell-targeted delivery of IL-2. This study also challenged a long-held paradigm of type 1 and 2 cytokine antagonism by newly identifying IL-13$^+$ IFN-γ$^+$ CD8$^+$ T cells that may potentially promote inflammation in SLE.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by SUNY UPSTATE IRB. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

Both authors participated in the design and analysis of the experiments and writing of the manuscript. HK executed the experiments. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by grants R01-AI072648 and AI122176 from the National Institutes of Health and the Department of Medicine at the SUNY Upstate Medical University.

ACKNOWLEDGMENTS

We thank all patients and healthy donors who contributed to this study.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.635531/full#supplementary-material

Supplementary Figure 1 | IL-2 does not control IL-4 expression. **(A)** CD4$^+$ and CD8$^+$ T cells from matched SLE and HC subjects were cultured as described in **Figure 2** and IL-13 and IL-4 expression was determined by flow cytometry. **(B)** Cumulative data of MFI and frequency of IL-4$^+$ cells. Statistical analysis was made by two-way ANOVA followed by Bonferroni's correction for multiple comparisons (*p<0.05).

Supplementary Figure 2 | CD4$^+$ T cells produce greater amount of IL-21 than CD8$^+$ T cells in an IL-2-independent manner. **(A)** CD4$^+$ and CD8$^+$ T cells from matched SLE and HC subjects were cultured as described in **Figure 2** and IL-13 and IL-21 expression was determined by flow cytometry. **(B)** Cumulative data of MFI and frequency of IL-21$^+$ cells. Statistical analysis was made by two-way ANOVA followed by Bonferroni's correction for multiple comparisons (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

Supplementary Figure 3 | Expression of IL-13 positively correlates with that of IL-5 and IFN-γ. **(A)** CD4$^+$ and CD8$^+$ T cells from matched SLE and HC subjects were cultured as described in **Figure 2**. The MFI of IL-13 was compared between IL-5$^+$ and IL-5$^-$ cells, IFN-γ$^+$ and IFN-γ$^-$ cells, and IL-4$^+$ and IL-4$^-$ cells. Data were analyzed by a paired two-tailed t-test (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001). **(B)** Pearson's and Spearman's correlation analyses were performed to determine the association between IL-13 and IL-5 or IFN-γ. Pearson correlation coefficient was presented for the association of IL-13 with IL-5. Spearman correlation coefficient was presented for the association of IL-13 with IFN-γ.

REFERENCES

1. Tsokos GC. Systemic lupus erythematosus. *N Engl J Med* (2011) 365:2110–21. doi: 10.1056/NEJMra1100359
2. Bonelli M, Savitskaya A, von Dalwigk K, Steiner CW, Aletaha D, Smolen JS, et al. Quantitative and qualitative deficiencies of regulatory T cells in patients with systemic lupus erythematosus (SLE). *Int Immunol* (2008) 20:861–8. doi: 10.1093/intimm/dxn044
3. Mellor-Pita S, Citores MJ, Castejon R, Tutor-Ureta P, Yebra-Bango M, Andreu JL, et al. Decrease of regulatory T cells in patients with systemic lupus erythematosus. *Ann Rheum Dis* (2006) 65:553–4. doi: 10.1136/ard.2005.044974
4. Miyara M, Amoura Z, Parizot C, Badoual C, Dorgham K, Trad S, et al. Global natural regulatory T cell depletion in active systemic lupus erythematosus. *J Immunol* (2005) 175:8392–400. doi: 10.4049/jimmunol.175.12.8392
5. Burchill MA, Yang J, Vogtenhuber C, Blazar BR, Farrar MA. IL-2 receptor beta-dependent STAT5 activation is required for the development of Foxp3+

regulatory T cells. *J Immunol* (2007) 178:280–90. doi: 10.4049/jimmunol.178.1.280
6. Linker-Israeli M, Bakke AC, Kitridou RC, Gendler S, Gillis S, Horwitz DA. Defective production of interleukin 1 and interleukin 2 in patients with systemic lupus erythematosus (SLE). *J Immunol* (1983) 130:2651–5.
7. 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
8. Morgan DA, Ruscetti FW, Gallo R. Selective in vitro growth of T lymphocytes from normal human bone marrows. *Science* (1976) 193:1007–8. doi: 10.1126/science.181845
9. 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
10. Shi M, Lin TH, Appell KC, Berg LJ. Janus-kinase-3-dependent signals induce chromatin remodeling at the Ifng locus during T helper 1 cell differentiation. *Immunity* (2008) 28:763–73. doi: 10.1016/j.immuni.2008.04.016
11. Pipkin ME, Sacks JA, Cruz-Guilloty F, Lichtenheld MG, Bevan MJ, Rao A. Interleukin-2 and inflammation induce distinct transcriptional programs that promote the differentiation of effector cytolytic T cells. *Immunity* (2010) 32:79–90. doi: 10.1016/j.immuni.2009.11.012
12. Kato H, Perl A. Mechanistic target of rapamycin complex 1 expands Th17 and IL-4+ CD4-CD8- double-negative T cells and contracts regulatory T cells in systemic lupus erythematosus. *J Immunol* (2014) 192:4134–44. doi: 10.4049/jimmunol.1301859
13. Lai ZW, Borsuk R, Shadakshari A, Yu J, Dawood M, Garcia R, et al. Mechanistic target of rapamycin activation triggers IL-4 production and necrotic death of double-negative T cells in patients with systemic lupus erythematosus. *J Immunol* (2013) 191:2236–46. doi: 10.4049/jimmunol.1301005
14. Crispin JC, Oukka M, Bayliss G, Cohen RA, Van Beek CA, Stillman IE, et al. Expanded double negative T cells in patients with systemic lupus erythematosus produce IL-17 and infiltrate the kidneys. *J Immunol* (2008) 181:8761–6. doi: 10.4049/jimmunol.181.12.8761
15. Blanco P, Pitard V, Viallard JF, Taupin JL, Pellegrin JL, Moreau JF. Increase in activated CD8+ T lymphocytes expressing perforin and granzyme B correlates with disease activity in patients with systemic lupus erythematosus. *Arthritis Rheum* (2005) 52:201–11. doi: 10.1002/art.20745
16. Winchester R, Wiesendanger M, Zhang HZ, Steshenko V, Peterson K, Geraldino-Pardilla L, et al. Immunologic characteristics of intrarenal T cells: trafficking of expanded CD8+ T cell beta-chain clonotypes in progressive lupus nephritis. *Arthritis Rheum* (2012) 64:1589–600. doi: 10.1002/art.33488
17. Horwitz DA, Gray JD, Behrendsen SC, Kubin M, Rengaraju M, Ohtsuka K, et al. Decreased production of interleukin-12 and other Th1-type cytokines in patients with recent-onset systemic lupus erythematosus. *Arthritis Rheum* (1998) 41:838–44. doi: 10.1002/1529-0131(199805)41:5<838::AID-ART10>3.0.CO;2-S
18. Akahoshi M, Nakashima H, Tanaka Y, Kohsaka T, Nagano S, Ohgami E, et al. Th1/Th2 balance of peripheral T helper cells in systemic lupus erythematosus. *Arthritis Rheum* (1999) 42:1644–8. doi: 10.1002/1529-0131(199908)42:8<1644::AID-ANR12>3.0.CO;2-L
19. Min DJ, Cho ML, Cho CS, Min SY, Kim WU, Yang SY, et al. Decreased production of interleukin-12 and interferon-gamma is associated with renal involvement in systemic lupus erythematosus. *Scand J Rheumatol* (2001) 30:159–63. doi: 10.1080/030097401300162932
20. Aversa G, Punnonen J, Cocks BG, de Waal Malefyt R, Vega FJr., Zurawski SM, et al. An interleukin 4 (IL-4) mutant protein inhibits both IL-4 or IL-13-induced human immunoglobulin G4 (IgG4) and IgE synthesis and B cell proliferation: support for a common component shared by IL-4 and IL-13 receptors. *J Exp Med* (1993) 178:2213–8. doi: 10.1084/jem.178.6.2213
21. Defrance T, Carayon P, Billian G, Guillemot JC, Minty A, Caput D, et al. Interleukin 13 is a B cell stimulating factor. *J Exp Med* (1994) 179:135–43. doi: 10.1084/jem.179.1.135
22. McKenzie AN, Culpepper JA, de Waal Malefyt R, Briere F, Punnonen J, Aversa G, et al. Interleukin 13, a T-cell-derived cytokine that regulates human monocyte and B-cell function. *Proc Natl Acad Sci USA* (1993) 90:3735–9. doi: 10.1073/pnas.90.8.3735
23. Miyahara N, Takeda K, Kodama T, Joetham A, Taube C, Park JW, et al. Contribution of antigen-primed CD8+ T cells to the development of airway hyperresponsiveness and inflammation is associated with IL-13. *J Immunol* (2004) 172:2549–58. doi: 10.4049/jimmunol.172.4.2549
24. Wills-Karp M, Luyimbazi J, Xu X, Schofield B, Neben TY, Karp CL, et al. Interleukin-13: central mediator of allergic asthma. *Science* (1998) 282:2258–61. doi: 10.1126/science.282.5397.2258
25. Fuschiotti P, Larregina AT, Ho J, Feghali-Bostwick C, Medsger TA Jr. Interleukin-13-producing CD8+ T cells mediate dermal fibrosis in patients with systemic sclerosis. *Arthritis Rheum* (2013) 65:236–46. doi: 10.1002/art.37706
26. Medsger TA Jr, Ivanco DE, Kardava L, Morel PA, Lucas MR, Fuschiotti P. GATA-3 up-regulation in CD8+ T cells as a biomarker of immune dysfunction in systemic sclerosis, resulting in excessive interleukin-13 production. *Arthritis Rheum* (2011) 63:1738–47. doi: 10.1002/art.30489
27. Tan EM, Cohen AS, Fries JF, Masi AT, McShane DJ, Rothfield NF, et al. The 1982 revised criteria for the classification of systemic lupus erythematosus. *Arthritis Rheum* (1982) 25:1271–7. doi: 10.1002/art.1780251101
28. Bombardier C, Gladman DD, Urowitz MB, Caron D, Chang CH. Derivation of the SLEDAI. A disease activity index for lupus patients. The Committee on Prognosis Studies in SLE. *Arthritis Rheum* (1992) 35:630–40. doi: 10.1002/art.1780350606
29. 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.
30. 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 Rheumatol* (2018) 70:427–38. doi: 10.1002/art.40380
31. Yamane H, Zhu J, Paul WE. Independent roles for IL-2 and GATA-3 in stimulating naive CD4+ T cells to generate a Th2-inducing cytokine environment. *J Exp Med* (2005) 202:793–804. doi: 10.1084/jem.20051304
32. Lai ZW, Kelly R, Winans T, Marchena I, Shadakshari A, Yu J, et al. Sirolimus in patients with clinically active systemic lupus erythematosus resistant to, or intolerant of, conventional medications: a single-arm, open-label, phase 1/2 trial. *Lancet* (2018) 391:1186–96. doi: 10.1016/S0140-6736(18)30485-9
33. Kim EH, Sullivan JA, Plisch EH, Tejera MM, Jatzek A, Choi KY, et al. Signal integration by Akt regulates CD8 T cell effector and memory differentiation. *J Immunol* (2012) 188:4305–14. doi: 10.4049/jimmunol.1103568
34. Krawczyk CM, Shen H, Pearce EJ. Functional plasticity in memory T helper cell responses. *J Immunol* (2007) 178:4080–8. doi: 10.4049/jimmunol.178.7.4080
35. Zhu J, Min B, Hu-Li J, Watson CJ, Grinberg A, Wang Q, et al. Conditional deletion of Gata3 shows its essential function in T(H)1-T(H)2 responses. *Nat Immunol* (2004) 5:1157–65. doi: 10.1038/ni1128
36. Yagi R, Junttila IS, Wei G, Urban JFJr., Zhao K, Paul WE, et al. The transcription factor GATA3 actively represses RUNX3 protein-regulated production of interferon-gamma. *Immunity* (2010) 32:507–17. doi: 10.1016/j.immuni.2010.04.004
37. 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
38. Bailis W, Yashiro-Ohtani Y, Fang TC, Hatton RD, Weaver CT, Artis D, et al. Notch simultaneously orchestrates multiple helper T cell programs independently of cytokine signals. *Immunity* (2013) 39:148–59. doi: 10.1016/j.immuni.2013.07.006

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.

frontiers in Immunology

MINI REVIEW
published: 09 April 2021
doi: 10.3389/fimmu.2021.662305

Ex-TFRs: A Missing Piece of the SLE Puzzle?

Xundong Wei[1,2], *Jianhua Zhang*[2,3] *and Xuyu Zhou*[2,3]*

[1] Center of Biotherapy, Beijing Hospital, National Center of Gerontology, Institute of Geriatric Medicine, Chinese Academy of Medical Sciences, Beijing, China, [2] Savaid Medical School, University of Chinese Academy of Sciences, Beijing, China, [3] CAS Key Laboratory of Pathogenic Microbiology and Immunology, Institute of Microbiology, Chinese Academy of Sciences (CAS), Beijing, China

Systemic lupus erythematosus (SLE) is a chronic multi-organ autoimmune disease involving the production of a wide range of autoantibodies and complement activation. The production of these high-affinity autoantibodies requires T cell/B cell collaboration as well as germinal center (GC) formation. T follicular regulatory cells (TFRs) are functional specialized T regulatory cells (Tregs) that safeguard against both self-reactive T and B cells. However, recent evidence suggests that TFRs are not always stable and can lose Foxp3 expression to become pathogenic "ex-TFRs" that gain potent effector functions. In this review, we summarize the literature on intrinsic and extrinsic mechanisms of regulation of TFR stability and discuss the potential role of TFR reprogramming in autoantibody production and SLE pathogenesis.

Keywords: systemic lupus erythematosus, T regulatory cell, ex-TFRs, Foxp3, stability, autoantibodies

OPEN ACCESS

Edited by:
Ciriaco A. Piccirillo, McGill University, Canada

Reviewed by:
Carlo Chizzolini, Université de Genève, Switzerland
Florencia Rosetti, Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán (INCMNSZ), Mexico

***Correspondence:**
Xuyu Zhou
zhouxy@im.ac.cn

Specialty section:
This article was submitted to Autoimmune and Autoinflammatory Disorders, a section of the journal Frontiers in Immunology

Received: *01 February 2021*
Accepted: *18 March 2021*
Published: *09 April 2021*

Citation:
Wei X, Zhang J and Zhou X (2021) Ex-TFRs: A Missing Piece of the SLE Puzzle? Front. Immunol. 12:662305. doi: 10.3389/fimmu.2021.662305

INTRODUCTION

Systemic lupus erythematosus (SLE) is a chronic multi-organ autoimmune disease with wide clinical heterogeneity. The disease can cause injury to many organs, especially the kidneys, joints, and skin. The pathogenesis of SLE is not fully understood, but increased high-affinity self-antibody production and dysregulated immune tolerance have been implicated in the progression of the disease (1, 2). SLE often starts in late childhood or adolescence and predominantly affects females in their reproductive years, with a female/male ratio of 9:1; the reasons for this skewed sex ratio remain unclear (3, 4). In the 1950s, only 50% of SLE patients survived for 5 years; now, due to early diagnosis and better treatment, most patients survive for more than 10 years. There is no effective treatment for SLE, and only a few drugs have been approved in the past 60 years, emphasizing the need for a better understanding of its pathogenesis.

A hallmark of SLE is the production of a wide range of autoantibodies by self-reactive B cells. Anti-nuclear antibodies (ANAs) are detected in >95% of SLE patients, and subsequent deposition of immune complexes in endogenous tissue results in severe tissue damage and induction of inflammation (5, 6). The autoantibodies from lupus patients are high affinity, somatic mutated, and class switched, and their generation requires the formation of germinal centers (GCs) with assistance from follicular helper T cells (TFHs) (7). In addition to their involvement in GC formation, TFHs, a unique $CD4^+$ subset of T cells with high expression of Bcl6, PD-1, and CXCR5,

play a major role in the selection of high-affinity B cells. TFHs have thus emerged as a critical immunoregulator of antibody production as well as the pathogenesis of human SLE (8).

Another small population of $CD4^+$ T cells, regulatory T cells (Tregs), maintain self-tolerance by suppressing both autoreactive T and B lymphocytes through the production of inhibitory cytokines such as IL-10, TGF-β, and IL-35 (9, 10). Similar to conventional T cells, TCR and MHC and peptide engagement will lead to the activation of Tregs and which contribute to the further development of functional specialized T follicular regulatory cells (TFRs). TFRs also express both Bcl6 and CXCR5 and are capable of traveling to B cell follicles to serve as gatekeepers controlling autoantibody production (11–13). Tregs were initially considered a stable lineage, but emerging evidence suggests that even fully committed Treg cells can lose their identity and be reprogrammed to effector T cells (14–17). Interestingly, reprogramming of Tregs has been observed in the follicular region. TFRs can lose their Foxp3 expression and become pathogenic "ex-TFRs" (18). Whether the autoreactive potential of ex-TFRs contributes to autoimmune disease is not known. In this review, we summarize recent progress in understanding the roles of signaling pathways and transcriptional and epigenetic regulation in modulating Treg and TFR stability. We also discuss the possibility that pathogenic ex-TFRs contribute to autoantibody production and the pathogenesis of SLE.

TFRS

In 1995, Sakaguchi et al. identified a small subset of $CD4^+$ T cells that express the high-affinity IL-2 receptor IL-2Rα (CD25) and are capable of suppressing autoimmunity upon transfer (19, 20). Indeed, mice lacking either IL-2Rα or IL-2 develop severe systemic autoimmunity (21–23). The cells identified by Sakaguchi et al. are now known as Tregs, and in 2003, the transcription factor Forkhead Box P3 (Foxp3) was identified as the lineage-defining regulator of Tregs (24–26). The importance of Foxp3 has been illustrated by studies of Foxp3 gene mutations, immune dysregulation, polyendocrinopathy, enteropathy, and X-linked (IPEX) syndrome in humans, and Scurfy mutant mice bearing Foxp3 mutations develop lethal multi-organ autoimmunity (27–30). In addition, ablation of Foxp3 in mature Tregs or depletion of $Foxp3^+$ cells completely eliminates the suppressive capacity of Tregs and programs Tregs into pathogenic T cells (31).

Tregs are not a homogenous population. Depending on their developmental origin, Tregs can be divided into thymic Tregs (tTregs) and peripheral Tregs (pTregs) (32, 33). tTregs are induced in the thymus and are characterized by high-affinity self-antigen engagement (34). By contrast, pTregs are generated from conventional $CD4^+$ T cells under conditions of high levels of transforming growth factor β (TGF-β) and retinoic acid in the environment or in response to metabolites produced by microbiota in the gut (35). Although the TCR repertoires of pTregs and tTregs overlap, tTregs mainly recognize self-antigens, whereas pTregs also express TCRs specific for non–self-infectious antigens or innocuous commensal microbiota derived antigens; these latter TCRs are important for the maintenance of mucosal tolerance (36, 37).

Similar to conventional T cells, Treg TCR engagement is the first step in generating heterogeneous effector Tregs, which are functionally potent and capable of migrating to local tissue (38). Effector Tregs can differentiate into specialized subsets by adapting the same set of transcription factors that control the differentiation of helper $CD4^+$ T cells. For example, the Th1 transcription factor T-bet drives the expression of CXCR3 in Tregs, which is important for regulating some Th1-mediated autoimmune responses (39), and the RORγt-expressing Treg is involved in the regulation of Th17-mediated experimental autoimmune encephalomyelitis (EAE) and colitis (40–42).

Bcl6-expressing TFR cells (TFRs) are a particularly important subset of effector Tregs that express CXCR5 and migrate to B cell follicles and GCs (11). TFRs are capable of modulating B cell responses and, given their unique localization, appear to be major players in controlling autoantibody production (43–48). Indeed, Treg-specific ablation of Bcl6 results in substantial increases in multiple autoreactive antibodies, including anti-dsDNA (49, 50). Sage et al. demonstrated the importance of TFR in controlling the production of a panel of self-antibodies in an elegant TFR-DTR model established by crossing Foxp3-Cre mouse with a CXCR5 floxed stop DTR (diphtheria toxin receptor) strain (12, 51). In this model, only cells expressing both Foxp3 and CXCR5 (such as TFRs) express DTR on the cell surface, making them susceptible to deletion upon administration of diphtheria toxin (DT).

TFRs differentiate from natural Treg precursors through interaction with dendritic cells (DCs) and require different costimulatory activation signals at different stages of differentiation. Treg cells do not express CXCR5 in the T-cell zone and only start to upregulate CXCR5 when they migrate to the border region between the T and B cell zones. These cells are defined as pre-TFRs (52). The early differentiation of pre-TFRs requires CD28 and ICOS helper signals from DC cells and is independent of B cells. Although the initial stage of TFR differentiation does not rely on B cells, the stable mature TFR program requires B cell assistance. B cell-deficient mice exhibit a large decrease in mature TFRs in the lymph nodes (47). In follicles, CD25 and Blimp-1 expression are downregulated in $CD25^+$ TFRs, leading to the acquisition of the $CXCR5^{hi}Bcl6^{hi}$ phenotype, which allows these $CD25^-$ TFRs to traffic into the GC (**Table 1**). Sage and colleagues demonstrated that TFRs prevent self-reactive B cells from being activated by TFHs, most likely *via* attenuated production of cytokines (such as IL-21 and IL-4) and/or costimulatory signals (53, 54). TFRs also prevent GC formation caused by foreign antigens (vaccines, microorganisms) by inhibiting the metabolic flux of B cells and through CTLA-4-mediated inhibition of B cells. TFRs may physically interrupt bidirectional costimulation and linked recognition during immunological synapses between TFHs and B cells (55). A specific subtype of TFHs, $SOSTDC1^+$ TFHs, promote TFR cell differentiation by inhibiting the β-catenin pathway through the secreted protein SOSTDC1 (56).

TABLE 1 | The markers for the identification of TFR and TFH.

Subtype	Foxp3	CD25	Blimp-1	CXCR5	PD-1	ICOS	Bcl6	CD44	CD62L	Location	Autoreactive
Naive Treg	+	+	–	–	–	–	–	–	+	Extrafollicle	High
Pre-TFR	++	++	+	+	+	+	+/-	+	–	T-B border	High
CD25$^+$TFR	++	++	++	++	++	++	++	+	–	Follicule	High
CD25$^-$TFR	++	–	–	+++	+++	+++	+++	+	–	GC	High
Ex-TFR	–	–	–	+++	+++	+++	+++	+	–	GC	High
Naive T	–	–	–	–	–	–	–	–	+	Extrafollicle	Low
Pre-TFH	–	–	–	+	+	+	+/-	+	–	T-B border	Low
TFH	–	–	–	++	++	++	++	+	–	Follicule	Low
GC-TFH	–	–	–	+++	+++	+++	+++	+	–	GC	Low

Defects in Treg function and/or number, particularly the TFR subset, are thought to contribute to SLE pathogenesis, but conflicting results have been reported. Some groups have found an increase in TFRs in SLE patients compared with healthy controls (57–59), whereas others have found reduced numbers or impaired function of circulating TFRs or Tregs in SLE patients (60, 61). Other groups have observed no abnormalities (62, 63). These discrepancies are due in part to the lack of a unique marker or combination of markers for identifying and isolating bona fide Tregs, the use of different *in vitro* stimuli, and the presence or absence of antigen-presenting cells (APCs) in *ex vivo* functional assays (64). An important challenge in the study of the pathogenesis of SLE is the difficulty of obtaining patient lymphoid tissues to assess TFRs directly; for this reason, most studies have focused on circulating Tregs in peripheral blood.

FOXP3 STABILITY OF TFRS

Tregs were initially considered a stable cell lineage committed to immunosuppressive function, but accumulating evidence indicates that they can lose Foxp3 expression and undergo reprogramming to other types of effector T cells. Upon transfer into CD3e KO mice, Foxp3$^+$CD4$^+$ T cells terminate Foxp3 expression and differentiate into TFHs in Peyer's patches (14). CD25$^-$Foxp3$^+$ T cells are likely more unstable than cells expressing CD25. In a fate-mapping experiment involving Foxp3 bacterial artificial chromosome (BAC) transgenic mice expressing GFP-Cre under the control of the Foxp3 promoter, we demonstrated that a fraction of Tregs are not stable. These "ex-Tregs", which no longer express Foxp3, have an activated-memory T cell phenotype and the ability to produce inflammatory cytokines such as IFN-γ and IL-17. Importantly, ex-Tregs bearing the BDC2.5 TCR induce autoimmune diabetes upon adoptive transfer (15). Autoimmune inflammation exacerbates the instability of Foxp3. By using MOG tetramer to identify antigen-specific Tregs, we further demonstrated that Tregs can be converted into pathogenic T helper cells in an EAE mouse model, suggesting a link between strong TCR signaling and Treg instability (16).

As a major TGF-β sensor, conserved noncoding sequence 1 (CNS1) in Foxp3 is critical for the generation of induced pTregs but dispensable for tTreg development. Given the heterogeneity of Tregs, we further generated a delta CNS1 Foxp3 BAC transgenic mouse strain that only traces committed and stable tTregs (17). We found that resting or naïve tTregs are stable, but upon development to TFRs, these cells can lose Foxp3 stability and be reprogrammed into a T helper lineage (17).

Sage et al. recently confirmed that a population of TFRs can lose Foxp3 expression in experiments using inducible Foxp3 fate-mapper mice (FoxP3$^{ERT2\text{-}Cre}$-Rosa26 Lox-Stop-Lox-TdTomato). In this model, Cre–ERT2 is limited to the cytoplasm in the absence of tamoxifen. Upon administration of tamoxifen, the tamoxifen metabolite 4-OHT (an analog of estrogen) binds to ERT, allowing Cre-ERT2 to enter the nucleus and exert Cre recombinase activity, thus triggering the expression of the fluorescent protein TdTomato in Foxp3$^+$Treg cells. In contrast to continuous labeling, inducible labeling of Foxp3$^+$Tregs with TdTomato during the immunization period avoids cell labeling due to transient Foxp3 expression and permits the assessment of bona fide Treg maintenance. Sage et al. immunized these mice with NP-OVA and 7 d later assessed the frequency of CXCR5$^+$CD4$^+$TdTomato$^+$Foxp3^{low} "ex-TFR" populations. In this model, ∽80% of CD4$^+$CXCR5$^+$TdTomato$^+$ cells retained Foxp3 (TFRs); the remaining ∽20% lost Foxp3 expression (ex-TFRs).

In summary, although TFRs are a functionally specialized subset of Tregs that selectively survey the autoreactive antibody response in the GC, continuous localization of TFRs in the GC might have a detrimental effect on Treg stability, leading to loss of Foxp3 expression and reprogramming to TFHs (**Figure 1**) (18).

WHY DO TFRS PREFERENTIALLY LOSE FOXP3?

The stability of Foxp3 expression is largely determined by the methylation status of the CNS2 region of the Foxp3 gene locus, which is also known as the Treg-specific demethylated region (TSDR) (65, 66). Foxp3 CNS2 contains 11 CpG sites, which are all methylated in peripheral conventional T cells as well as thymic DP and CD4 SP cells. Gradual demethylation of CNS2 occurs during tTreg development (67, 68); this process is not passively cell cycle dependent but is mediated by Tet-dependent oxidation, which is primarily mediated by Tet2 and Tet3 (69). Demethylation of CNS2 leads to the recruitment of transcription

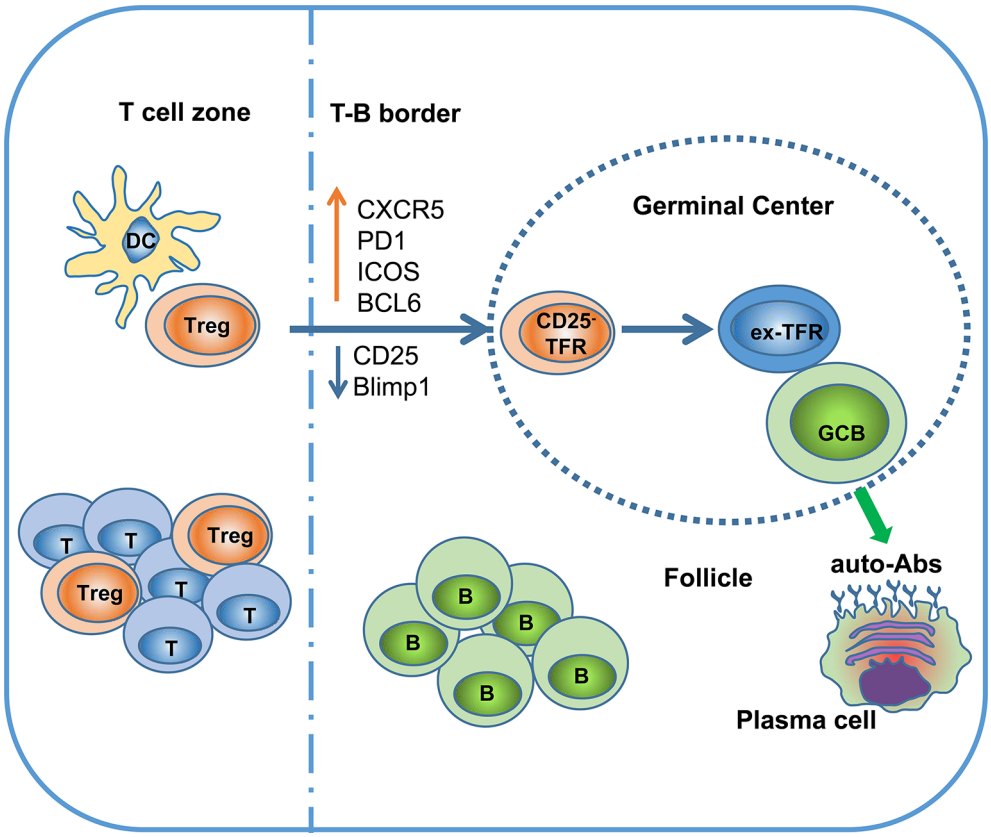


FIGURE 1 | Ex-TFRs: a missing piece of the SLE puzzle. Naïve Treg cells can interact with dendritic cells (DCs) to become activated and further migrate into the germinal center (GC) region through upregulation of CXCR5 and BCL6. In the follicle and GC, TFRs play an important role in regulating antigen (Ag)-specific TFHs and antibody-secreting cells. Since the GC is not favorable to stable Foxp3 expression, some TFRs will lose Foxp3 and develop into pathogenic TFHs (ex-TFRs). Ex-TFRs tend to recognize self-antigens, which may promote autoreactive humoral immunity.

factors such as Cbfβ, Runx1, STAT5, and Foxp3 itself, thereby reinforcing Foxp3 expression on Tregs (65, 70–72). Indeed, genetically deleting either the CNS2 enhancer of the Foxp3 locus or Tet family proteins leads to a destabilized Treg lineage and the development of spontaneous autoimmunity and chronic inflammation (73).

Treg stability is influenced by many intrinsic and extrinsic factors, particularly cytokines and their downstream signaling pathways. IL-2 and STAT5 activation maintain Foxp3 stability by binding directly to CNS2, and the Hippo kinases Mst1 and Mst2 promote STAT5 activation to further strengthen the Treg lineage (74, 75). By contrast, IL-4 and IL-6 can have detrimental effects on the Treg lineage. IL-4 receptor (IL-4R) knock-in mice in which IL-4R signaling is specifically upregulated exhibit reduced Treg stability and promotion of the Th2 response (76). STAT6 and STAT3, which are downstream of IL-4R, appear to compete with STAT5 at the CNS2 region of Foxp3. Depletion of SOSC1 ($Socs1^{fl/fl}$ × $Foxp3^{YFP\text{-}Cre}$ mice), a natural inhibitor of STAT proteins, destabilizes the Treg lineage, and more interestingly, adoptive transfer of SOSC1-deficient Tregs is sufficient to induce autoimmune colitis (77).

Although ablation of TCR in mature Tregs has little impact on Treg stability, overstimulation of Tregs *via* dysregulation of TCR and/or co-stimulation profoundly destabilizes the Treg lineage. A number of negative regulators of the TCR signaling pathway, such as PTEN, ITCH, Vhl, and PTPN, play important roles in maintaining the stability of the Treg lineage (78–80). Interestingly, Tregs themselves are partially anergic compared with conventional T cells. Under conditions of homeostasis, Tregs remain anergic, but TCR signaling upon weak stimulation confers strong suppressive potential on Tregs without reducing the stability of the lineage (81, 82). However, overstimulation causes Treg destabilization and reprogramming into pathogenic effector cells (17, 80, 83). The detailed molecular mechanism of the TCR signaling pathway has not been fully elucidated, but metabolic mechanisms could be very important; some metabolic pathways may interact with transcriptional and epigenetic regulation to modulate the Treg lineage.

Another important regulator of Treg lineage maintenance is the Foxp3 complex itself; roles of EzH2, RelA, and Runx, among other components of the complex, have been demonstrated (84, 85). Post-translational modification of the Foxp3 protein is part

of a feedback loop that controls Foxp3 stability. CRISPR-Cas9-based screening is beginning to reveal a more comprehensive picture of Treg lineage regulation, and new regulators such as Usp22, Rnf20, and Brd9 have been identified (86, 87). Interestingly, Zheng et al. found that non-canonical BAF (ncBAF) can localize at Foxp3 cis-regulatory elements to promote Foxp3 binding, whereas another SWI/SNF subunit, PBAF, seems to exert opposing effects (88).

In addition to its critical role in maintaining Foxp3 stability (89, 90), the IL-2/STAT5 signaling pathway is a potent negative regulator of TFH differentiation. IL-2 has been reported to repress TFR differentiation by a STAT5/Blimp-1 dependent mechanism (91). Thus, downregulation of the high-affinity IL-2 receptor CD25 is likely a common strategy for avoiding excessive STAT5 signaling in TFRs and TFHs. Consistent with this notion, CD25 expression is typically low or absent on TFHs and TFRs (92). By contrast, TFRs express high levels of inducible costimulator (ICOS), a co-stimulation molecule belonging to the CD28 family. ICOS signaling through the PI3K/AKT pathway is essential for the initiation of TFH and TFR differentiation and is also an important survival signal for $CD25^-$ effector Tregs (93–95). However, as mentioned above, such positive signals can dampen Treg stability; for example, loss of Blimp-1, a strong transcriptional repressor of Bcl6, boosts TFR differentiation but has a detrimental effect on Treg stability (96). Together, the CD25 and ICOS signal switch during TFR cell differentiation is the driving force for programming TFRs to become TFHs (96).This notion is also consistent with previous adoptive transfer experiments showing that $CD25^-Foxp3^+$ cells preferentially differentiate into effector TFHs under lymphopenic conditions (**Table 1**).

EX-TFRS: A MISSING PIECE OF THE SLE PUZZLE?

The functional role of ex-TFRs is not fully understood. The large majority of TFRs express Helios, a transcription factor expressed by tTregs (97), suggesting that TFRs are thymic in origin and biased toward self-antigens. This notion is further supported by recent studies indicating a highly diverse TCR repertoire of TFRs (36, 37). The loss of Foxp3 expression on TFRs generates a population of T cells with the potential to attack self-tissue. These cells could have a similar function as autoreactive TFHs. Sage et al. showed that ex-TFRs lose their suppressive function and have a transcriptional signature that is more similar to TFHs than TFRs (18). Moreover, multiple lines of Treg conditional knockout mice exhibit defects in maintenance of Treg stability, in association with an increased autoreactive humoral response and even the development of lupus-like autoimmune disorders (98–100). For example, mice in which PTEN is conditionally knocked out in Tregs develop a lupus-like autoimmune lymphoproliferative disease characterized by excessive levels of TFHs and B cell activation. These mice also exhibit increased serum levels of multiple auto-antibodies and creatinine, indicating renal pathology (80, 101). Tet2/3 conditional knockout mice develop lethal autoimmunity in association with the production of numerous self-antibodies (73), and a similar autoimmune disease is observed in $Foxp3Cre^{WT/Cre}Tet2/3^{fl/fl}$ heterozygous female mice, which harbor half of the wild type of Tregs in the same mice (73). These results strongly support the notion that ex-Tregs are self-recognition biased and have pathogenic potential (**Figure 1**).

Treg stability has not been directly tested in mouse models of lupus or human patients. However, Benoist et al. found that $Foxp3^+$ Treg cells are unstable in NZW mice, which may explain the reduced sensitivity of this NZW Tregs to limiting doses of trophic cytokines, IL-2 and -33 (102). In addition, this instability may provide a genetic explanation for disease pathogenesis, as NZW × NZB F1 female mice develop a severe autoimmune disease that shares many features of SLE in human patients (103).

CONCLUSIONS

SLE is an autoimmune disease characterized by the production of a wide range of autoantibodies, and its exact pathoetiology remains elusive. Although TFRs play a critical role in controlling autoantibody production, the migration of TFRs to the follicular region and GC does not favor stable Foxp3 expression, and some TFRs even lose Foxp3 and develop into TFHs with pathogenic potential. These ex-TFRs are likely biased toward self-recognition and might promote autoreactive humoral immunity. A better understanding of the role of ex-TFRs could have important therapeutic implications for SLE and many other autoimmune diseases.

AUTHOR CONTRIBUTIONS

XW, JZ, and XZ wrote the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the National Natural Science Foundation of China (31870911, 81802872), the National Science and Technology Major Project of China (2016ZX10004222-007 and 2018ZX10301-208-002-002).

REFERENCES

1. Yu C, Gershwin ME, Chang C. Diagnostic criteria for systemic lupus erythematosus: a critical review. *J Autoimmun* (2014) 48-49:10–3. doi: 10.1016/j.jaut.2014.01.004
2. Mohan C, Putterman C. Genetics and pathogenesis of systemic lupus erythematosus and lupus nephritis. *Nat Rev Nephrol* (2015) 11:329–41. doi: 10.1038/nrneph.2015.33
3. Harry O, Yasin S, Brunner H. Childhood-Onset Systemic Lupus Erythematosus: A Review and Update. *J Pediatr* (2018) 196:22–30 e2. doi: 10.1016/j.jpeds.2018.01.045

4. Feldman CH, Hiraki LT, Liu J, Fischer MA, Solomon DH, Alarcon GS, et al. Epidemiology and sociodemographics of systemic lupus erythematosus and lupus nephritis among US adults with Medicaid coverage, 2000-2004. *Arthritis Rheumatol* (2013) 65:753–63. doi: 10.1002/art.37795
5. Burbelo PD, Keller J, Kopp JB. New horizons for human pathogenic autoantibodies. *Discovery Med* (2015) 20:17–25.
6. Hamilton JA, Hsu HC, Mountz JD. Autoreactive B cells in SLE, villains or innocent bystanders? *Immunol Rev* (2019) 292:120–38. doi: 10.1111/imr.12815
7. Jenks SA, Cashman KS, Woodruff MC, Lee FE, Sanz I. Extrafollicular responses in humans and SLE. *Immunol Rev* (2019) 288:136–48. doi: 10.1111/imr.12741
8. Blanco P, Ueno H, Schmitt N. T follicular helper (Tfh) cells in lupus: Activation and involvement in SLE pathogenesis. *Eur J Immunol* (2016) 46:281–90. doi: 10.1002/eji.201545760
9. 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
10. Wei X, Zhang J, Gu Q, Huang M, Zhang W, Guo J, et al. Reciprocal Expression of IL-35 and IL-10 Defines Two Distinct Effector Treg Subsets that Are Required for Maintenance of Immune Tolerance. *Cell Rep* (2017) 21:1853–69. doi: 10.1016/j.celrep.2017.10.090
11. 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–E09. doi: 10.1073/pnas.1705551114
12. Sage PT, Sharpe AH. The multifaceted functions of follicular regulatory T cells. *Curr Opin Immunol* (2020) 67:68–74. doi: 10.1016/j.coi.2020.10.009
13. Wing JB, Lim EL, Sakaguchi S. Control of foreign Ag-specific Ab responses by Treg and Tfr. *Immunol Rev* (2020) 296:104–19. doi: 10.1111/imr.12888
14. 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
15. Zhou X, Bailey-Bucktrout SL, Jeker LT, Penaranda C, Martinez-Llordella M, Ashby M, et al. Instability of the transcription factor Foxp3 leads to the generation of pathogenic memory T cells in vivo. *Nat Immunol* (2009) 10:1000–7. doi: 10.1038/ni.1774
16. Bailey-Bucktrout SL, Martinez-Llordella M, Zhou X, Anthony B, Rosenthal W, Luche H, et al. Self-antigen-driven activation induces instability of regulatory T cells during an inflammatory autoimmune response. *Immunity* (2013) 39:949–62. doi: 10.1016/j.immuni.2013.10.016
17. Zhang Z, Zhang W, Guo J, Gu Q, Zhu X, Zhou X. Activation and Functional Specialization of Regulatory T Cells Lead to the Generation of Foxp3 Instability. *J Immunol* (2017) 198:2612–25. doi: 10.4049/jimmunol.1601409
18. Hou S, Clement RL, Diallo A, Blazar BR, Rudensky AY, Sharpe AH, et al. FoxP3 and Ezh2 regulate Tfr cell suppressive function and transcriptional program. *J Exp Med* (2019) 216:605–20. doi: 10.1084/jem.20181134
19. 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.
20. Itoh M, Takahashi T, Sakaguchi N, Kuniyasu Y, Shimizu J, Otsuka F, et al. Thymus and autoimmunity: production of CD25+CD4+ naturally anergic and suppressive T cells as a key function of the thymus in maintaining immunologic self-tolerance. *J Immunol* (1999) 162:5317–26.
21. Furtado GC, Curotto de Lafaille MA, Kutchukhidze N, Lafaille JJ. Interleukin 2 signaling is required for CD4(+) regulatory T cell function. *J Exp Med* (2002) 196:851–7. doi: 10.1084/jem.20020190
22. Nelson BH. Interleukin-2 signaling and the maintenance of self-tolerance. *Curr Dir Autoimmun* (2002) 5:92–112. doi: 10.1159/000060549
23. Mullins GN, Valentine KM, Al-Kuhlani M, Davini D, Jensen KDC, Hoyer KK. T cell signaling and Treg dysfunction correlate to disease kinetics in IL-2Ralpha-KO autoimmune mice. *Sci Rep* (2020) 10:21994. doi: 10.1038/s41598-020-78975-y
24. Fontenot JD, Gavin MA, Rudensky AY. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *Nat Immunol* (2003) 4:330–6. doi: 10.1038/ni904
25. 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
26. Khattri R, Cox T, Yasayko SA, Ramsdell F. An essential role for Scurfin in CD4+CD25+ T regulatory cells. *Nat Immunol* (2003) 4:337–42. doi: 10.1038/ni909
27. Bennett CL, Christie J, Ramsdell F, Brunkow ME, Ferguson PJ, Whitesell L, 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
28. Bennett CL, Ochs HD. IPEX is a unique X-linked syndrome characterized by immune dysfunction, polyendocrinopathy, enteropathy, and a variety of autoimmune phenomena. *Curr Opin Pediatr* (2001) 13:533–8. doi: 10.1097/00008480-200112000-00007
29. Brunkow ME, Jeffery EW, Hjerrild KA, Paeper B, Clark LB, Yasayko SA, et al. Disruption of a new forkhead/winged-helix protein, scurfin, results in the fatal lymphoproliferative disorder of the scurfy mouse. *Nat Genet* (2001) 27:68–73. doi: 10.1038/83784
30. Sawant DV, Vignali DA. Once a Treg, always a Treg? *Immunol Rev* (2014) 259:173–91. doi: 10.1111/imr.12173
31. Williams LM, Rudensky AY. Maintenance of the Foxp3-dependent developmental program in mature regulatory T cells requires continued expression of Foxp3. *Nat Immunol* (2007) 8:277–84. doi: 10.1038/ni1437
32. Bluestone JA, Abbas AK. Natural versus adaptive regulatory T cells. *Nat Rev Immunol* (2003) 3:253–7. doi: 10.1038/nri1032
33. Hori S. Lineage stability and phenotypic plasticity of Foxp3(+) regulatory T cells. *Immunol Rev* (2014) 259:159–72. doi: 10.1111/imr.12175
34. Jordan MS, Boesteanu A, Reed AJ, Petrone AL, Holenbeck AE, Lerman MA, et al. Thymic selection of CD4+CD25+ regulatory T cells induced by an agonist self-peptide. *Nat Immunol* (2001) 2:301–6. doi: 10.1038/86302
35. Atarashi K, Tanoue T, Shima T, Imaoka A, Kuwahara T, Momose Y, et al. Induction of colonic regulatory T cells by indigenous Clostridium species. *Science* (2011) 331:337–41. doi: 10.1126/science.1198469
36. 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
37. 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) 115:9604–09. doi: 10.1073/pnas.1808594115
38. Li MO, Rudensky AY. T cell receptor signalling in the control of regulatory T cell differentiation and function. *Nat Rev Immunol* (2016) 16:220–33. doi: 10.1038/nri.2016.26
39. Koch MA, Thomas KR, Perdue NR, Smigiel KS, Srivastava S, Campbell DJ. T-bet(+) Treg cells undergo abortive Th1 cell differentiation due to impaired expression of IL-12 receptor beta2. *Immunity* (2012) 37:501–10. doi: 10.1016/j.immuni.2012.05.031
40. Ayyoub M, Deknuydt F, Raimbaud I, Dousset C, Leveque L, Bioley G, et al. Human memory FOXP3+ Tregs secrete IL-17 ex vivo and constitutively express the T(H)17 lineage-specific transcription factor RORgamma t. *Proc Natl Acad Sci USA* (2009) 106:8635–40. doi: 10.1073/pnas.0900621106
41. Voo KS, Wang YH, Santori FR, Boggiano C, Wang YH, Arima K, et al. Identification of IL-17-producing FOXP3+ regulatory T cells in humans. *Proc Natl Acad Sci USA* (2009) 106:4793–8. doi: 10.1073/pnas.0900408106
42. Kim BS, Lu H, Ichiyama K, Chen X, Zhang YB, Mistry NA, et al. Generation of RORgammat(+) Antigen-Specific T Regulatory 17 Cells from Foxp3(+) Precursors in Autoimmunity. *Cell Rep* (2017) 21:195–207. doi: 10.1016/j.celrep.2017.09.021
43. 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
44. Sage PT, Sharpe AH. T follicular regulatory cells. *Immunol Rev* (2016) 271:246–59. doi: 10.1111/imr.12411
45. 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
46. 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

47. 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
48. 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
49. 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
50. Xie MM, Fang S, Chen Q, Liu H, Wan J, Dent AL. Follicular regulatory T cells inhibit the development of granzyme B-expressing follicular helper T cells. *JCI Insight* (2019) 4:e128076. doi: 10.1172/jci.insight.128076
51. Clement RL, Daccache J, Mohammed MT, Diallo A, Blazar BR, Kuchroo VK, et al. Follicular regulatory T cells control humoral and allergic immunity by restraining early B cell responses. *Nat Immunol* (2019) 20:1360–71. doi: 10.1038/s41590-019-0472-4
52. 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
53. 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
54. 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
55. 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
56. Wu X, Wang Y, Huang R, Gai Q, Liu H, Shi M, et al. SOSTDC1-producing follicular helper T cells promote regulatory follicular T cell differentiation. *Science* (2020) 369:984–88. doi: 10.1126/science.aba6652
57. 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
58. Jacquemin C, Augusto JF, Scherlinger M, Gensous N, Forcade E, Douchet I, et al. OX40L/OX40 axis impairs follicular and natural Treg function in human SLE. *JCI Insight* (2018) 3:e122167. doi: 10.1172/jci.insight.122167
59. 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) 56:261–68. doi: 10.1016/j.intimp.2018.01.038
60. Lyssuk EY, Torgashina AV, Soloviev SK, Nassonov EL, Bykovskaia SN. Reduced number and function of CD4+CD25highFoxP3+ regulatory T cells in patients with systemic lupus erythematosus. *Adv Exp Med Biol* (2007) 601:113–9. doi: 10.1007/978-0-387-72005-0_12
61. Valencia X, Yarboro C, Illei G, Lipsky PE. Deficient CD4+CD25high T regulatory cell function in patients with active systemic lupus erythematosus. *J Immunol* (2007) 178:2579–88. doi: 10.4049/jimmunol.178.4.2579
62. Alvarado-Sanchez B, Hernandez-Castro B, Portales-Perez D, Baranda L, Layseca-Espinosa E, Abud-Mendoza C, et al. Regulatory T cells in patients with systemic lupus erythematosus. *J Autoimmun* (2006) 27:110–8. doi: 10.1016/j.jaut.2006.06.005
63. Vargas-Rojas MI, Crispin JC, Richaud-Patin Y, Alcocer-Varela J. Quantitative and qualitative normal regulatory T cells are not capable of inducing suppression in SLE patients due to T-cell resistance. *Lupus* (2008) 17:289–94. doi: 10.1177/0961203307088307
64. Chavele KM, Ehrenstein MR. Regulatory T-cells in systemic lupus erythematosus and rheumatoid arthritis. *FEBS Lett* (2011) 585:3603–10. doi: 10.1016/j.febslet.2011.07.043
65. Zheng Y, Josefowicz S, Chaudhry A, Peng XP, Forbush K, Rudensky AY. Role of conserved non-coding DNA elements in the Foxp3 gene in regulatory T-cell fate. *Nature* (2010) 463:808–12. doi: 10.1038/nature08750
66. Li X, Liang Y, LeBlanc M, Benner C, Zheng Y. Function of a Foxp3 cis-element in protecting regulatory T cell identity. *Cell* (2014) 158:734–48. doi: 10.1016/j.cell.2014.07.030
67. Floess S, Freyer J, Siewert C, Baron U, Olek S, Polansky J, et al. Epigenetic control of the foxp3 locus in regulatory T cells. *PloS Biol* (2007) 5:e38. doi: 10.1371/journal.pbio.0050038
68. Ohkura N, Hamaguchi M, Morikawa H, Sugimura K, Tanaka A, Ito Y, et al. T cell receptor stimulation-induced epigenetic changes and Foxp3 expression are independent and complementary events required for Treg cell development. *Immunity* (2012) 37:785–99. doi: 10.1016/j.immuni.2012.09.010
69. Yue X, Trifari S, Aijo T, Tsagaratou A, Pastor WA, Zepeda-Martinez JA, et al. Control of Foxp3 stability through modulation of TET activity. *J Exp Med* (2016) 213:377–97. doi: 10.1084/jem.20151438
70. Kim HP, Leonard WJ. CREB/ATF-dependent T cell receptor-induced FoxP3 gene expression: a role for DNA methylation. *J Exp Med* (2007) 204:1543–51. doi: 10.1084/jem.20070109
71. Rudra D, Egawa T, Chong MM, Treuting P, Littman DR, Rudensky AY. Runx-CBFbeta complexes control expression of the transcription factor Foxp3 in regulatory T cells. *Nat Immunol* (2009) 10:1170–7. doi: 10.1038/ni.1795
72. Polansky JK, Schreiber L, Thelemann C, Ludwig L, Kruger M, Baumgrass R, et al. Methylation matters: binding of Ets-1 to the demethylated Foxp3 gene contributes to the stabilization of Foxp3 expression in regulatory T cells. *J Mol Med (Berl)* (2010) 88:1029–40. doi: 10.1007/s00109-010-0642-1
73. Yue X, Lio CJ, Samaniego-Castruita D, Li X, Rao A. Loss of TET2 and TET3 in regulatory T cells unleashes effector function. *Nat Commun* (2019) 10:2011. doi: 10.1038/s41467-019-09541-y
74. Shi H, Liu C, Tan H, Li Y, Nguyen TM, Dhungana Y, et al. Hippo Kinases Mst1 and Mst2 Sense and Amplify IL-2R-STAT5 Signaling in Regulatory T Cells to Establish Stable Regulatory Activity. *Immunity* (2018) 49:899–914 e6. doi: 10.1016/j.immuni.2018.10.010
75. Chen L. Non-canonical Hippo signaling regulates immune responses. *Adv Immunol* (2019) 144:87–119. doi: 10.1016/bs.ai.2019.07.001
76. Pelly VS, Coomes SM, Kannan Y, Gialitakis M, Entwistle LJ, Perez-Lloret J, et al. Interleukin 4 promotes the development of ex-Foxp3 Th2 cells during immunity to intestinal helminths. *J Exp Med* (2017) 214:1809–26. doi: 10.1084/jem.20161104
77. Takahashi R, Nakatsukasa H, Shiozawa S, Yoshimura A. SOCS1 Is a Key Molecule That Prevents Regulatory T Cell Plasticity under Inflammatory Conditions. *J Immunol* (2017) 199:149–58. doi: 10.4049/jimmunol.1600441
78. Jin HS, Park Y, Elly C, Liu YC. Itch expression by Treg cells controls Th2 inflammatory responses. *J Clin Invest* (2013) 123:4923–34. doi: 10.1172/JCI69355
79. Lee JH, Elly C, Park Y, Liu YC. E3 Ubiquitin Ligase VHL Regulates Hypoxia-Inducible Factor-1alpha to Maintain Regulatory T Cell Stability and Suppressive Capacity. *Immunity* (2015) 42:1062–74. doi: 10.1016/j.immuni.2015.05.016
80. 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
81. Levine AG, Arvey A, Jin W, Rudensky AY. Continuous requirement for the TCR in regulatory T cell function. *Nat Immunol* (2014) 15:1070–8. doi: 10.1038/ni.3004
82. Vahl JC, Drees C, Heger K, Heink S, Fischer JC, Nedjic J, et al. Continuous T cell receptor signals maintain a functional regulatory T cell pool. *Immunity* (2014) 41:722–36. doi: 10.1016/j.immuni.2014.10.012
83. Huynh A, DuPage M, Priyadharshini B, Sage PT, Quiros J, Borges CM, et al. Control of PI(3) kinase in Treg cells maintains homeostasis and lineage stability. *Nat Immunol* (2015) 16:188–96. doi: 10.1038/ni.3077
84. DuPage M, Chopra G, Quiros J, Rosenthal WL, Morar MM, Holohan D, et al. The chromatin-modifying enzyme Ezh2 is critical for the maintenance of regulatory T cell identity after activation. *Immunity* (2015) 42:227–38. doi: 10.1016/j.immuni.2015.01.007
85. Samstein RM, Arvey A, Josefowicz SZ, Peng X, Reynolds A, Sandstrom R, et al. Foxp3 exploits a pre-existent enhancer landscape for regulatory T cell lineage specification. *Cell* (2012) 151:153–66. doi: 10.1016/j.cell.2012.06.053
86. Cortez JT, Montauti E, Shifrut E, Gatchalian J, Zhang Y, Shaked O, et al. CRISPR screen in regulatory T cells reveals modulators of Foxp3. *Nature* (2020) 582:416–20. doi: 10.1038/s41586-020-2246-4

87. Cameron J, Martino P, Nguyen L, Li X. Cutting Edge: CRISPR-Based Transcriptional Regulators Reveal Transcription-Dependent Establishment of Epigenetic Memory of Foxp3 in Regulatory T Cells. *J Immunol* (2020) 205:2953–58. doi: 10.4049/jimmunol.2000537
88. Loo CS, Gatchalian J, Liang Y, Leblanc M, Xie M, Ho J, et al. A Genome-wide CRISPR Screen Reveals a Role for the Non-canonical Nucleosome-Remodeling BAF Complex in Foxp3 Expression and Regulatory T Cell Function. *Immunity* (2020) 53:143–57 e8. doi: 10.1016/j.immuni.2020.06.011
89. Fontenot JD, Rasmussen JP, Gavin MA, Rudensky AY. A function for interleukin 2 in Foxp3-expressing regulatory T cells. *Nat Immunol* (2005) 6:1142–51. doi: 10.1038/ni1263
90. Burchill MA, Yang J, Vogtenhuber C, Blazar BR, Farrar MA. IL-2 receptor beta-dependent STAT5 activation is required for the development of Foxp3+ regulatory T cells. *J Immunol* (2007) 178:280–90. doi: 10.4049/jimmunol.178.1.280
91. 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
92. 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
93. 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
94. 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
95. 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.1700409
96. Shen E, Rabe H, Luo L, Wang L, Wang Q, Yin J, et al. Control of Germinal Center Localization and Lineage Stability of Follicular Regulatory T Cells by the Blimp1 Transcription Factor. *Cell Rep* (2019) 29:1848–61 e6. doi: 10.1016/j.celrep.2019.10.012
97. 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
98. Zhou X, Jeker LT, Fife BT, Zhu S, Anderson MS, McManus MT, et al. Selective miRNA disruption in T reg cells leads to uncontrolled autoimmunity. *J Exp Med* (2008) 205:1983–91. doi: 10.1084/jem.20080707
99. Muto G, Kotani H, Kondo T, Morita R, Tsuruta S, Kobayashi T, et al. TRAF6 is essential for maintenance of regulatory T cells that suppress Th2 type autoimmunity. *PloS One* (2013) 8:e74639. doi: 10.1371/journal.pone.0074639
100. Liu B, Salgado OC, Singh S, Hippen KL, Maynard JC, Burlingame AL, et al. The lineage stability and suppressive program of regulatory T cells require protein O-GlcNAcylation. *Nat Commun* (2019) 10:354. doi: 10.1038/s41467-019-08300-3
101. Wu D, Luo Y, Guo W, Niu Q, Xue T, Yang F, et al. Lkb1 maintains Treg cell lineage identity. *Nat Commun* (2017) 8:15876. doi: 10.1038/ncomms15876
102. Depis F, Kwon HK, Mathis D, Benoist C. Unstable FoxP3+ T regulatory cells in NZW mice. *Proc Natl Acad Sci USA* (2016) 113:1345–50. doi: 10.1073/pnas.1524660113
103. Elouaai F, Lule J, Benoist H, Appolinaire-Pilipenko S, Atanassov C, Muller S, et al. Autoimmunity to histones, ubiquitin, and ubiquitinated histone H2A in NZB x NZW and MRL-lpr/lpr mice. Anti-histone antibodies are concentrated in glomerular eluates of lupus mice. *Nephrol Dial Transplant* (1994) 9:362–6.

Conflict of Interest: XW consulted for Zhongke Qiyuan (Shenzhen) Biotechnology Co., Ltd. in T cell immunity.

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.

frontiers in Immunology

REVIEW
published: 14 April 2021
doi: 10.3389/fimmu.2021.629807

Harnessing Tolerogenic Histone Peptide Epitopes From Nucleosomes for Selective Down-Regulation of Pathogenic Autoimmune Response in Lupus (Past, Present, and Future)

Syamal K. Datta *

Department of Medicine, Division of Rheumatology, Northwestern University Feinberg School of Medicine, Chicago, IL, United States

Autoantigen-directed tolerance can be induced by certain nucleosomal histone peptide epitope/s in nanomolar dosage leading to sustained remission of disease in mice with *spontaneous* SLE. By contrast, lupus is *accelerated* by administration of intact (whole) histones, or whole nucleosomes in microparticles from apoptotic cells, or by post-translationally acetylated histone-peptides. Low-dose therapy with the histone-peptide epitopes simultaneously induces TGFβ and inhibits IL-6 production by DC *in vivo*, especially pDC, which then induce CD4+CD25+ Treg and CD8+ Treg cells that suppress pathogenic autoimmune response. Both types of induced Treg cells are FoxP3+ and act by producing TGFβ at close cell-to-cell range. No anaphylactic adverse reactions, or generalized immunosuppression have been detected in mice injected with the peptides, because the epitopes are derived from evolutionarily conserved histones in the chromatin; and the peptides are expressed in the thymus during ontogeny, and their native sequences have not been altered. The peptide-induced Treg cells can block severe lupus on adoptive transfer reducing inflammatory cell reaction and infiltration in the kidney. In *Humans*, similar potent Treg cells are generated by the histone peptide epitopes *in vitro* in *lupus patients'* PBMC, inhibiting anti-dsDNA autoantibody and interferon production. Furthermore, the same types of Treg cells are generated in lupus patients who are in very long-term remission (2-8 years) after undergoing autologous hematopoietic stem cell transplantation. These Treg cells are not found in lupus patients treated conventionally into clinical remission (SLEDAI of 0); and consequently they still harbor pathogenic autoimmune cells, causing subclinical damage. Although antigen-specific therapy with pinpoint accuracy is suitable for straight-forward organ-specific autoimmune diseases, Systemic Lupus is much more complex. The histone peptide epitopes have unique tolerogenic properties for inhibiting Innate immune cells (DC), T cells and B cell populations that are both antigen-specifically and cross-reactively involved in the pathogenic autoimmune response in lupus. The histone peptide tolerance is a natural and non-toxic therapy suitable for treating early lupus, and

OPEN ACCESS

Edited by:
Shohei Hori,
The University of Tokyo, Japan

Reviewed by:
Keishi Fujio,
The University of Tokyo, Japan
Gilberto Filaci,
University of Genoa, Italy

****Correspondence:***
Syamal K. Datta
skd257@northwestern.edu

Specialty section:
This article was submitted to Immunological Tolerance and Regulation, a section of the journal Frontiers in Immunology

Received: *16 November 2020*
Accepted: *23 March 2021*
Published: *14 April 2021*

Citation:
Datta SK (2021) Harnessing Tolerogenic Histone Peptide Epitopes From Nucleosomes for Selective Down-Regulation of Pathogenic Autoimmune Response in Lupus (Past, Present, and Future). Front. Immunol. 12:629807. doi: 10.3389/fimmu.2021.629807

also maintaining lupus patients after toxic drug therapy. The experimental steps, challenges and possible solutions for successful therapy with these peptide epitopes are discussed in this highly focused review on Systemic Lupus.

Keywords: autoimmunity, systemic lupus erythematosus, T regulatory cells, T suppressor cells, autoantigen specific tolerance, autoantigen derived peptide epitopes

INTRODUCTION

This chronicle with historical perspective focuses first on the early steps of pathogenic autoantibody production in lupus, especially on the role of Th cells, and then how they can be regulated. In human lupus, it is now established that the main genetic risk loci for lupus susceptibility in GWAS are located in MHC class II and IRF5 regions, which respectively determine autoantigen presentation and associated activating cytokines production required to recruit autoreactive T helper (Th) cells (1–8). Genetic studies in families with rheumatic autoimmune diseases also support the initiating role of T cells in lupus (9), in addition to complement genes in the MHC locus (10). Importantly, augmented Th cell activity which is prevalent in lupus (11–13), can overcome the need for TLR abnormalities contributing to lupus (14). B cells and other professional APCs are activated to present autoantigens as the disease progresses. However, normally macrophages, such as, tingible body MΦ and DCs remain tolerogenic when handling dying (apoptotic) cells that can provide the autoantigens for lupus if mishandled, as described below (15–18). The professional APC become effectively activated *in vivo* to present these apoptotic autoantigens after the apoptotic cell derived DNA and/or RNA containing autoantigens are presented in IgG immune complexes (IC) that are bound by the APC to dually stimulate their TLR and FcγR (19, 20). Hence, Th cell mediated class-switched IgG autoantibodies specific for the DNA or RNA containing autoantigens have to be made first for IC formation activating the APC. Moreover, B cells become efficient antigen presenter to lupus Th cells that have been primed first by other APC, or if the B cells have developed high affinity receptors after undergoing somatic mutation and expansion with T_{FH} cell help in germinal centers (19, 21). However, high level expression of X-linked TLR7, due to incomplete X-chromosome inactivation (22), can contribute to lupus development early on, by independently activating DC and other APC, which in turn causes widespread T-cell activation (23, 24). To accomplish the above effect, striking studies have recently shown that IRF5 is first activated by TLR7 using the adaptor TASL, which interacts with SLC15A4, an amino acid transporter in endolysosome, to recruit IRF5 (25). The X-inked gene CXorf21-a encoding TASL and the gene for SLC15A4 were known to be associated with lupus susceptibility, as discussed in ref (26). Of course intrinsic defects in B cells and APC are critically important for lupus pathogenesis. With disease progression, other pathogenic players in T cell, B cell and unconventional APC populations evolve and are recruited to participate in amplifying the autoimmune inflammatory response, especially in extra-follicular sites, to cite a few (27–32), and reviewed elsewhere [Tsokos, 2020 #2492] (33, 34). Those pathogenic contributors might be kept in check by establishing regulatory mechanisms at the earliest steps of the disease, which is the focus of this review on Lupus, and this topic.

IDENTIFYING AND CLONING PATHOGENIC ANTI-DSDNA AUTOANTIBODY-INDUCING TH CELLS OF LUPUS IN PATIENTS AND LUPUS-PRONE MICE (HISTORICAL PERSPECTIVE)

Step by step experiments and ensuing hypothesis based on their results at each stage led to cellular and molecular characterization of the pathogenic Th cells of lupus and how the Th cells become capable in helping pathogenic autoantibody production.

Properties of Pathogenic Anti-DNA Autoantibodies

First of all, certain distinctive properties of pathogenic anti-DNA autoantibodies were crucial for isolating and characterizing the Th cells that specifically help them. The pathogenic anti-dsDNA autoantibodies that are deposited in kidneys with lupus nephritis have distinct features, as they are complement-fixing IgG in isotypes, with cationic charge, and clonally restricted by isoelectric focusing, and are able to cause glomerulonephritis *in vivo* (35–41). Moreover, their antigen combining V regions share recurrent idiotype and fine-specificity patterns for autoantigens (39, 42). Sequence analysis of the pathogenic autoantibodies confirmed their clonal expansion, as they shared V_H region CDR3 sequences containing numerous cationic residues generated by somatic mutation (43–45), a signature of Th cell drive. Contemporary studies had shown that immune complexes with cationic charge preferentially bind to anionic residues in glomerular basement membrane proteoglycans and collagen (46–48). It was shown later that glomerular binding of these "anti-DNA" antibodies could also be mediated *via* histones in nucleosomes bound *in situ* (49–52).

Initial Studies to Find the Link Determining Cognate Interaction Between Autoimmune T and B Cells of Lupus

As described above, pathogenic anti-dsDNA antibodies in lupus are class-switched (35, 36) and clonally expanded (43, 44) suggesting a T helper cell dependent response, but it was

mysterious up to 1980s and early 1990s how the Th cells actually helped Pathogenic anti-dsDNA autoantibody-producing B cells, because conventional Th cells do not recognize DNA.

In the first step, it was established that special autoimmune T helper (Th) cell subsets expanded the select population of pathogenic anti-dsDNA autoantibody producing B cells in mice with lupus (41, 53). The production of these pathogenic autoantibodies is also driven by select Th cells that are detectable in patients with active lupus nephritis (54–56).

In the next step, to define their antigenic specificity, the autoimmune Th cells were cloned from lupus prone mice, and also from patients with lupus nephritis (~ 800 clones). Prior to these studies, isolation of the pathogenic T helper (Th) cells of lupus was not done, because their antigenic specificities were unknown. However, using their special functional property of inducing the pathogenic variety of anti-dsDNA autoantibodies as a selection marker, the lupus nephritis-inducing Th cells were isolated. Only 12-15% of activated T cells in lupus patients and mouse models, could induce the production of pathogenic IgG anti-DNA autoantibodies (53–57). When administered into young pre-clinical stage lupus-prone mice, pathogenic autoantibody-inducing Th clones could rapidly induce immune-deposit glomerulonephritis (57, 58). Sequences of antigen-binding CDR loops of the TCRs of these pathogenic Th clones of lupus show recurrent motifs of anionic residues, indicating their selection by autoantigens with cationic residues (56, 57, 59). Indeed, a majority of such pathogenic Th clones produced IL-2 and IFN-γ in response to nucleosomes that contain histone peptides bearing cationic determinants, and nucleosome-specific T cells are detectable in pre-clinical stage lupus-prone mice before pathogenic autoantibodies are detectable in their serum (56, 60, 61). In addition, immunization of pre-clinical lupus mice, but not normal mice, with whole nucleosome particles induces accelerated lupus nephritis indicating the need for pre-existing autoimmune T and B cells in a lupus-prone background (60).

Thus the relevant autoantigens for pathogenic anti-dsDNA autoantibody inducing Th cells of lupus were discovered by an unbiased experimental approach, using pathogenic autoantibody inducing Th cells as *sensors* to detect the relevant autoantigen epitopes. This property provided the lupus Th cells the ability of "linked recognition" (62) for interaction with pathogenic anti-DNA autoantibody producing B cells (**Figure 1**). In this way, for the first time a true autoantigen for spontaneous SLE; namely endogenous nucleosomes from host's apoptotic cells, and not some speculative component in microbes (63, 64), was found to be the real text book-like hapten- carrier link between the pathogenic Th and B cell in lupus for cognate interaction (57, 60),—and from that critical experimental step further studies led to identification of the histone peptide epitopes in nucleosomes recognized by those Th cells, and showed how to harness those particular epitopes for regulatory T cell induction for lupus therapy, as described below. All this was possible in 1980s and early 1990s by cloning the select population of pathogenic anti-dsDNA autoantibody-producing B cells, and then the special autoimmune T helper cells that drive such B cells in lupus. To

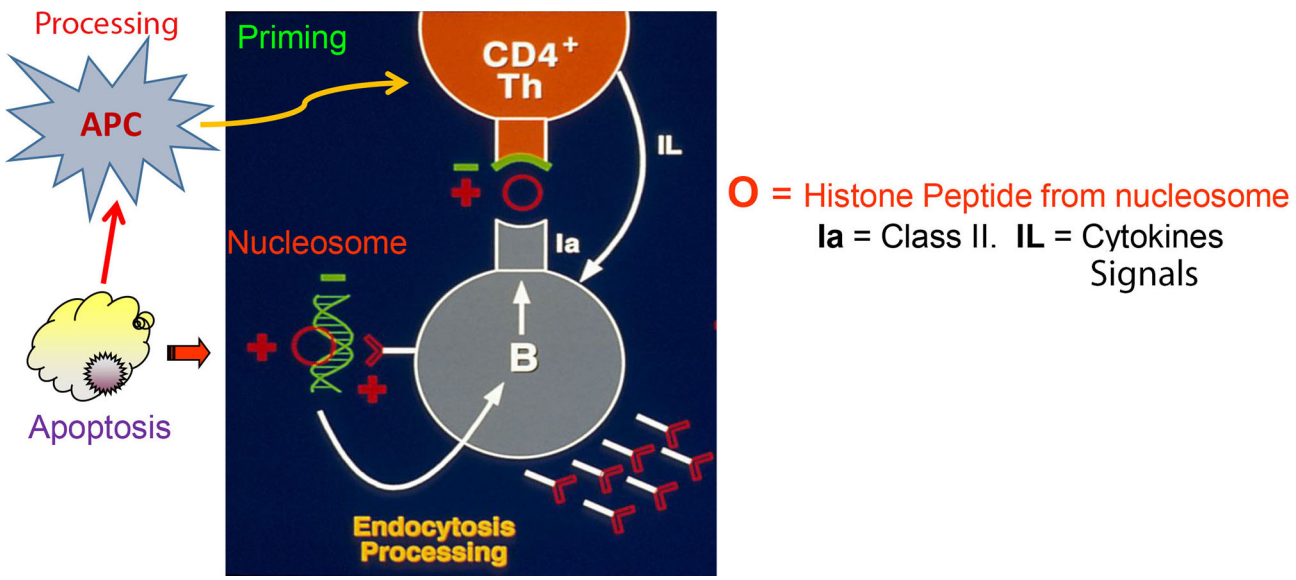


FIGURE 1 | Autoimmune T and B cell interaction in lupus based on Nucleosome-derived autoantigens (based on work done from early 1980s through early1990s; references in the Text). Figure shows that Th cells that induce the production of pathogenic anti-DNA autoantibodies possess anionic residues in CDR3 of their TCRs (green). The lupus Th cells recognize peptides with reciprocal cationic charge (red), such as peptides from histones in nucleosomes presented by the pathogenic anti-DNA autoantibody producing B cells bearing BCRs with cationic residues generated by somatic mutations in CDR3 of their receptors (red). The pathogenic BCRs bind to anionic residues in DNA (green) that are complexed with cationic histones in nucleosomes, which are then endocytosed and processed for presentation to the interacting Th cells. Nucleosomes accumulate due to defective clearance of apoptotic cells in lupus, and are processed by activated APC to prime the pathogenic Th cells; a lupus-specific event initiated early in life. This figure is extensively modified from a figure in J Exp Med (60).

emphasize again, despite the obstacle that the antigenic specificities of lupus T cells were then unknown, using the experimental steps described above, the pathogenic anti-dsDNA autoantibody-inducing T helper cells were cloned to define the structure and specificity of their receptor genes in human and murine lupus. To achieve the ultimate goal of understanding the cause, and designing a cure for spontaneous autoimmune diseases like lupus, it was essential at that time to identify the major autoantigen/s that drives the pathogenic autoimmune response. In lupus, DNA is a target antigen for autoantibodies but paradoxically immunization of mice with DNA does not cause lupus. The studies with the pathogenic autoantibody-inducing T helper clones in early 1990s led to the initial identification of one of the major immunogens that drives the pathogenic T helper cells of lupus (57, 60).

Significance and Relevant Contemporary Studies by Others

Many studies soon followed that demonstrated or suggested mechanisms that could initiate or amplify the response of pathogenic Th cells to nucleosomal peptides in lupus. Briefly, in lupus, products from apoptotic cells accumulate and become immunogenic because, scavenging molecules in phagocytic cells, such as Marco and other scavenger receptors are functionally deficient in lupus, and so are complement components such as C1q, which facilitate phagocytosis of apoptotic cells without causing an immune response (65–68). Nucleosomes, HMGB1, DNA or RNA from apoptotic cell components not being disposed of properly, act as endogenous TLR ligands, stimulating cells of the innate and adaptive immune system (19, 23, 60, 69–74). For example, HMGB1 chromosomal protein from apoptotic cells that have not been removed properly, forms inflammatory complexes with other accumulating debris like DNA or nucleosomes particles, stimulating immune cells *via* TLR 2, TLR 4, and RAGE on the cell-surface, or TLR9 in the endosome/lysosome (74–76). Similarly, accumulating extra-cellular nucleosomes in micro particles containing DNA, or ribonucleoproteins containing RNA can stimulate cells of the innate immune system respectively by TLR9 or by TLR 7/8 (29, 31, 77, 78), thus augmenting autoantigen presentation to pathogenic Th cells by those activated APC. The case for TLR 9 is actually more complex, because in early stages, TLR9 actually protects against lupus (79), possibly by promoting tolerance in APC, B cells and helping Treg generation (80, 81). The other possible autoantigen-derived epitope with cationic charge, which could be recognized in a lupus B cell-linked fashion by the pathogenic autoantibody inducing Th cells possessing reciprocally charged anionic residues in their CDR3 region, would be derived from CDR3 region peptides of somatically mutated anti-DNA autoantibodies, as suggested (57, 60); and this possibility was independently demonstrated to be true by several laboratories (82–85). This issue is dealt in other contributions to this research topic.

How Are the Many Types of Th Cells of Lupus Linked?

Th1, T_{FH}, T_{PH}, Gamma Delta Th, CD8 Th, $CD4^-CD8^-$ Th cells and more, participate in contributing to the pathogenic response in lupus. However, is it mainly Th1 → T_{FH} cells initiating/sustaining pathogenic autoantibody production; whereas the others evolve as amplifiers at extrafollicular inflammatory sites? First of all as mentioned above, only particular subclasses of IgG anti-DNA antibodies are more closely associated with a pathogenic potential in lupus patients and mice, and these pathogenic IgG antibodies belong to Th1-induced isotype classes. In lupus patients, Th1-induced anti-DNA IgG1antibodies are always elevated before the occurrence of renal relapse, and IgG1 plus IgG2 anti-DNA antibodies are found in patient's renal eluates, whereas in lupus prone mice, murine Th1-induced IgG2a, IgG2b, and IgG3 anti-DNA are more frequently eluted from kidneys with active nephritis (39, 42, 86–88). In contrast to T_{FH} cells which conventionally produce IL-21; Th1 cytokine IFNγ not only mediates class switch for the nephritogenic isotypes, but Th1 derived IFNγ signal is also critical for autoantibody production by germinal center B cells (89, 90). Furthermore, many non-autoantigen specific, bystander T_{FH} cells expand as a secondary event with progression of disease, which could amplify (but not initiate) anti-DNA autoantibody production (91). Indeed, Ig class-switch recombination (CSR), during T and B cell cognate interaction, which is Th1 IFNγ cytokine dependent in lupus, may occur before the T_{FH} IL-21 driven expansion of autoantibody producing B cells in germinal center, which comes later (92). Moreover, *Th1-biased GC T_{FH} cells* have been reported (93), and another group reported that the differentiation and function of a *Th1-derived T_{FH}1-like* cell population is driven by IL-12 signaling, which is important for differentiation of Th1 cells in the first place (94–96).

Therefore, *Th1 → T_{FH}1 evolution/transition* is a possibility in pathogenic anti-DNA autoantibody production in lupus.

And then there are the potent T_{PH} cells with T_{FH} like phenotype but are $CXCR5^-$; they help lupus B cells also by producing IL-21; and IL-10–producing $CCR6^+$T cells populate lymph nodes of SLE patients. These Th cells probably evolve after receiving cytokine and other signals from activated B cells and other APC at extra-follicular sites, as the disease progresses (27, 97).

In addition, helper activity of $CD8^+$ and $CD4^-/CD8^-$ αβ and γδ TCR^+ Th cells, in pathogenic autoantibody production in human SLE has been reported (54, 55). The subset of T cells in humans that are $CD4^-/CD8^-$ and αβ TCR^+ with pathogenic anti-DNA autoantibody-inducing ability in SLE is interesting, because such Th cells were considered to be unique to MRL-lpr mice with lupus. However, similar pathogenic autoantibody-inducing T cells with double negative phenotype that express "forbidden", autoreactive T cell receptors were described in non-lpr lupus prone mice (41, 53, 98) and then in human lupus (54, 55). Although these double negative T cells might be secondary events in lupus compared to the CD4+ Th cells, they make an important contribution to pathogenesis of the disease. The $CD4^-/CD8^-$ and αβ TCR^+ T cells also have important role in target organ inflammation (99, 100).

Nucleosomal Peptide Autoepitopes Recognized by Pathogenic Th and B Cells of Lupus

In the next step, the critical peptide autoepitopes recognized by lupus nephritis-inducing Th cells were localized initially to be in the core histones of nucleosomes, at amino acid (aa) positions:

10-33 of H-2B, 16-39 and 71-94 of H4, and 85-102 of H3 (61). Altogether 154 overlapping 15-mer peptides spanning the entire length of all four core histones were tested to find the buried epitopes in nucleosomal histones that were recognized specifically autoimmune Th cells that cause lupus in mouse models. In addition, another dominant epitope was identified in position 22-42 of H1', by mass spectrometry analysis of naturally processed peptides eluted from class II molecules of lupus B cell (APC) lines fed with chromatin (101). Remarkably, all these epitopes are located in regions of histones that contact with DNA in the nucleosome, and they are also targeted by autoantibodies from lupus B cells (B-cell epitopes), indicating that the epitopes could be protected from degradation during autoantigen processing and thus preferentially presented to the Th cells (61, 101–103). Surprisingly, the nucleosomal peptides have the features of "universal epitopes" (104), for instance, the peptide epitopes are promiscuously recognized by pathogenic Th cells derived from lupus-prone SNF_1 mice (MHC I-$A^{d/q}$) even when presented by APC bearing I-A molecules of all other mouse haplotypes, and human HLA-DR as well! Due to reciprocal charge interaction, the lupus TCRs probably contact the nucleosomal peptide-complexed with MHC promiscuously to sustain TCR signaling (105, 106). The promiscuity of lupus TCRs influences their selection in the thymus of lupus-prone mice and ability to generate Treg cells for tolerance spreading in the periphery, as described below (80, 107–109).

Nested Epitopes for CD8 T Cells. The tolerogenic nucleosomal peptide autoepitopes bind to MHC class II as described above, but $CD8^+$ Treg cells were also induced by injection of the epitopes. Indeed algorithms showed, MHC class I-binding motifs were nested in their sequences, as described (108, 110). The rationale being that the relatively long chain peptides epitopes would be processed further by APC for cross-presentation to CD8 T cells (111). For an example, $H4_{71–94}$ nucleosomal epitope has the nested CD8 sequence shown in bold letters TYTEHAKRKTVTAM DVVYALKRQG, and similarly individually distinct nested CD8 epitopes were detected in each of the longer peptide epitopes from the nucleosomes with CD4 Treg inducing ability, as detailed (108).

TOLERANCE THERAPY WITH NUCLEOSOMAL PEPTIDE EPITOPES

Generation of Autoepitope Specific CD4 Treg and CD8 Treg Cell Subsets in Lupus by Low-Dose Tolerance Therapy With Nucleosomal Histone Peptides

(Experimental Steps of More Recent Publications in Mouse Models and Then in Human Lupus Are Described in Brief Below):

Studies in Lupus Prone Mouse Models

(a). Publication Title: "Very Low Dose Tolerance With Nucleosomal Peptides Controls Lupus and Induces Potent Regulatory T Cell Subsets"

The major autoepitopes for lupus nephritis-inducing Th cells were localized to $H1'_{22-42}$, $H3_{85-102}$, $H4_{16-39}$ and $H4_{71-94}$, as described above. These peptide epitopes stimulate both autoimmune Th cells and B cells. In lupus-prone mice, tolerance therapy at High doses (300μg I.V.) of the peptide epitopes halted the progression of established lupus nephritis. However, high-dose may not be suitable in humans. Therefore, low-dose tolerance therapy was developed with 300 fold lower doses by injecting lupus-prone mice with 1 μg nucleosomal histone peptide autoepitopes S.C. every 2 wk (108). This sub-nanomolar peptide therapy lowered autoantibody levels, blocked nephritis progression and markedly diminished inflammatory cell infiltration in kidneys, thus restoring normal life span. $H4_{71-94}$ was the most effective autoepitope in this study. Low-dose tolerance therapy induced regulatory cell subsets of $CD8^+$ suppressor Treg, and $CD4^+CD25^+$ Treg cells, which contained autoantigen-specific and cross-reactive autoantigen-directed Treg cells. The Treg cells suppressed IFN-γ production by pathogenic lupus Th cells in response to nucleosomal epitopes at up to 1:100 ratio of Treg or Treg : Th cells, and diminished autoantibody production *in vitro* by up to 90-100% by inhibiting nucleosome-stimulated T cell help to nuclear autoantigen-specific B cells. The induced $CD4^+CD25^+$ Treg and $CD8^+$ Treg cells produced, and required TGF-β1 for immunosuppression; moreover, they effectively suppressed lupus autoimmunity upon adoptive transfer *in vivo*. For their suppressor function, the $CD4^+CD25^+$ Treg cells were partially cell contact-dependent, but $CD8^+$ Treg cells were contact-independent. Thus, this work demonstrated that low-dose tolerance with the conserved histone autoepitopes durably ameliorates the regulatory defect in SLE by inducing TGF-β producing Treg cells, and without causing adverse side effects such as, generalized immunosuppression or allergic/anaphylactic reactions (**Figures 2** and **3**).

(b). Publication Title: "Low-Dose Peptide Tolerance Therapy of Lupus Generates Tolerogenic Plasmacytoid Dendritic Cells That Cause Expansion of Autoantigen-Specific Treg Cells Along With Contraction of Inflammatory Th1 and Th17 Cell Populations"

As noted above, low-dose tolerance of mice with lupus using just a single nucleosomal peptide epitope ($H4_{71-94}$) could halt the progression of lupus nephritis by generating potent Treg cells that suppressed autoimmune T and B cells specific for a broad spectrum of nuclear autoantigens, and markedly inhibited inflammatory cell reaction and infiltration in kidneys. Next step was to determine how this therapy with only 0.36 nM of peptide injected subcutaneously (S.C.) every 2 weeks, induced *in vivo* TGFβ-producing CD8+Treg, and CD4+25+ Treg cell subsets containing regulatory cells that were autoantigen-specific, as established by the following approach (80). In order to track which APC had captured the histone peptide after tolerance therapy; DC, macrophages and B cells were isolated from local lymph nodes and spleens of lupus-prone mice injected with low-dose $H4_{71-94}$ peptide S.C., and then those APC were tested for their ability to stimulate cognate $H4_{71-94}$-specific T cell hybridomas in culture. The T cell hybridomas are highly sensitive and specific sensors detecting cognate peptide-MHC II on APCs presenting attomole concentration of the histone peptide. Only DC and B cells from spleen of histone peptide-

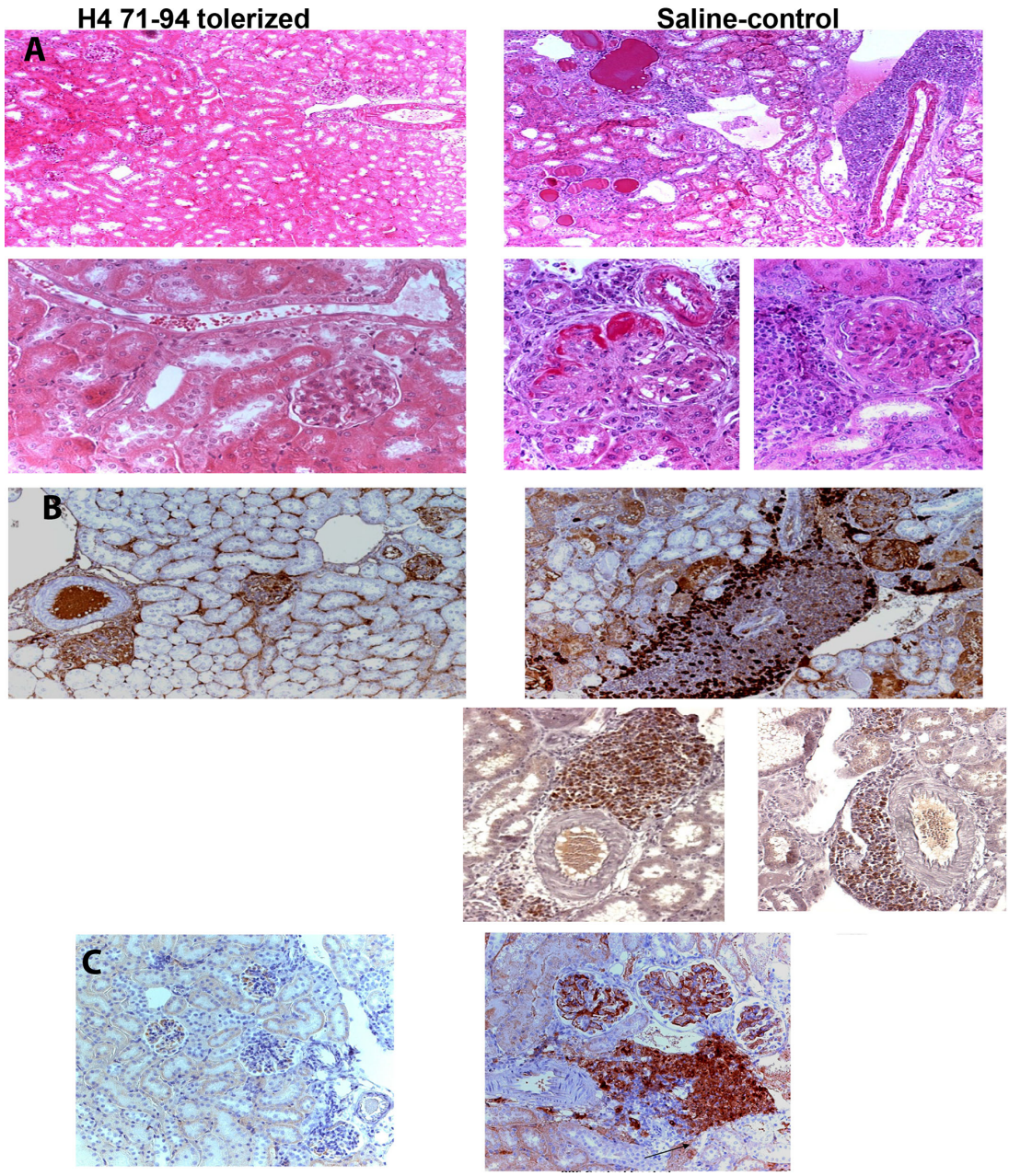


FIGURE 2 | **(A)** Renal histology from lupus prone mice tolerized by histone peptide epitope (*left*) or age-matched saline-injected control mice (*right*). H&E staining; ×100 magnification shown in *Upper panels*. The saline control shows marked interstitial infiltrate of mononuclear cells with perivascular distribution, hyalinized and sclerotic glomeruli and tubules engorged with casts. *Lower panels* (original magnification, ×400) show in further detail the differences between mice that underwent peptide-epitope therapy (*left*) and control mice (*right*). Kidneys from the former group of mice show marked thickening of basement membranes and advanced sclerosis and crescent formation in glomeruli, and perivascular, interstitial infiltrates of mononuclear cells. **(B)** Immunohistochemistry (original magnification was ×200). Brown color shows positive staining for IgG deposits in glomeruli of lupus-prone mice, in both peptide-treated (*left upper panel*) and control groups (*right upper panel*). However, marked cellular infiltrates around blood vessels containing $CD4^+$ T cells (on the *right, in upper panel*), $CD8^+$ T cells (on *left in lower panel*), and $CD138^+$ plasma cells (on *right side in lower panel*) were found only in kidneys of control mice, although both groups had IgG immune complex deposits. **(C)** Immunohistochemistry showing glomerular, and interstitial-perivascular infiltration of Th17 cells in control (PBS)-injected control lupus mice (*Right side*). This inflammatory cell infiltration was prevented in age-matched control mice by low-dose tolerance therapy with nucleosomal histone peptide epitope (*Left Panel*). Figure partially derived from J Immunol (80, 108), (Originally published in *The Journal of Immunology*. Kang H-K, Michaels MA, Berner BR, Datta SK. Very low-dose tolerance with nucleosomal peptides controls lupus and induces potent regulatory T cell subsets. *J Immunol* (2005) 174:3247-55. And Kang H-K, Liu M, Datta SK. Low-Dose Peptide Tolerance Therapy of Lupus Generates Plasmacytoid Dendritic Cells That Cause Expansion of Autoantigen-Specific Regulatory T Cells and Contraction of Inflammatory Th17 Cells *J Immunol* (2007) 178:7849-58. Copyright © [2005 and 2007] The American Association of Immunologists, Inc.).

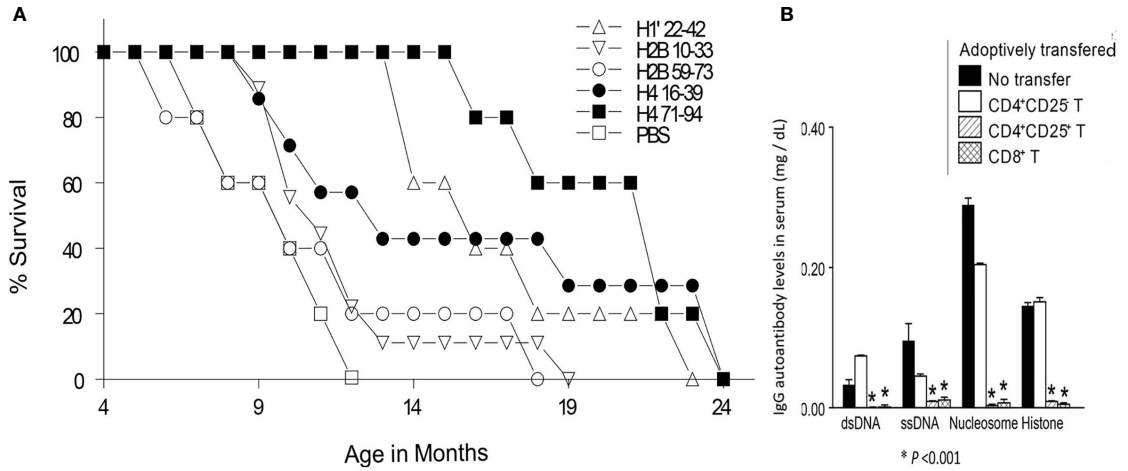


FIGURE 3 | **(A)** Percent survival, of age-matched, lupus-diseased mice, injected subcutaneously with different nucleosomal histone peptides in low-doses, or with saline (PBS) every two week. **(B)** In a rigorous test for potency of suppression, Treg cells from $H4_{71-94}$ peptide tolerized mice suppressed lupus acceleration upon adoptive transfer *in vivo*. Pre-clinical lupus-prone mice were *immunized* (not tolerized) by another histone peptide at 100 fold *higher doses* with *adjuvant* CFA, leading them to produce augmented levels of autoantibodies and develop severe nephritis rapidly, and this accelerated disease was suppressed by adoptive transfer of CD4+CD25+ Treg and CD8+Treg cells, but not CD4+CD25$^-$ effector T cells from $H4_{71-94}$-treated tolerized mice. *Y-axis* values are for IgG autoantibody levels in serum of recipients (mg/dL). *P <0.001. Parts of this Figure are from J Immunol (80, 108). Experimental details are in those references. (Originally published in *The Journal of Immunology*. Kang H-K, Michaels MA, Berner BR, Datta SK. Very low-dose tolerance with nucleosomal peptides controls lupus and induces potent regulatory T cell subsets. *J Immunol* (2005) 174:3247-55. And Kang H-K, Liu M, Datta SK. Low-Dose Peptide Tolerance Therapy of Lupus Generates Plasmacytoid Dendritic Cells That Cause Expansion of Autoantigen-Specific Regulatory T Cells and Contraction of Inflammatory Th17 Cells *J Immunol* (2007) 178:7849-58.

injected mice stimulated the T hybridomas. Thus, during tolerance therapy, the subcutaneously injected $H4_{71-94}$ peptide, which is highly soluble and charged, was rapidly absorbed systemically and captured by APC in the spleen. However, splenic DC, but not B cells or macrophages was responsible for the tolerogenic effect of the peptide therapy. Adoptive transfer of plasmacytoid DC or whole DC, but not B cells from the $H4_{71-94}$ peptide treated mice suppressed responses of autoimmune T cells to nucleosome peptides up to 80% by Treg cells induced in the un-manipulated lupus mouse recipients, and blocked development of nephritis and autoantibody production in a lupus acceleration assay. The DC from the $H4_{71-94}$ peptide injected mice expressed a tolerogenic phenotype upon capturing the S.C. injected $H4_{71-94}$ peptide, expressing relatively low levels of CD80, CD86, CD40 and MHC class II. Compared to controls, the peptide epitope treated animal's DC, especially plasmacytoid DC (pDC) produced increased amounts of TGFβ but decreased amount of IL-6 on stimulation by nucleosomes and other TLR-ligands, surprisingly the TLR-9 pathway was important for this tolerogenic effect (80). Moreover, these $H4_{71-94}$ peptide-tolerized pDC ameliorated lupus autoimmune disease by simultaneously inducing/expanding contained autoantigen-specific and cross-reactive autoantigen-directed Treg, and suppressing effector Th1 and Th17 cells that infiltrate the kidneys causing lupus nephritis. As an aside, these studies initially showed that inflammatory Th17 infiltrate the kidneys of mice with lupus nephritis (80), which was then demonstrated also in human lupus nephritis (99, 112, 113).

Altogether, these studies early on showed the pathogenic importance of tubulo-interstitial region infiltration in lupus nephritis kidneys by various inflammatory cells in addition to monocyte/macrophages; such as extrafollicular germinal center like accumulation of CD4 and CD8 T cells, and B cells and plasma cells to set up residence in organized perivascular foci, as well as Th17 cells; and importantly, this infiltration was inhibited by the histone peptide epitope tolerance therapy resulting in its beneficial effect (80, 108) (**Figure 2**). As discussed below, similar to these therapeutic results, the role of locally active Treg cells migrating into the kidney and suppressing lupus nephritis has been recently demonstrated in other systems (114–116). Interestingly, **Figure 2B**, shows that IgG immune complex deposits were equally present in the kidneys in both peptide-treated and control lupus-prone mice, but interstitial infiltrates of interacting T and B cells and APC were prominent only in the control mice with severe nephritis. This observation is consistent with the demonstration that lupus B cells can contribute to nephritis even without autoantibody production, but just by autoantigen presentation and providing cytokine and other membrane signals to pathogenic Th cells (117); and that Belimumab has beneficial effect in patients with active lupus nephritis (118), which is surprising, but we now know that mature memory B cells also express BAFFR like immature transitional B cells (119). All these intricate pathogenic

interactions were prevented by tolerance therapy with the histone peptide epitopes (**Figure 2**).

SIGNIFICANCE OF ABOVE STUDIES IN LUPUS-PRONE MICE

Cross-Reactive Recognition of Nuclear Autoantigens of Lupus and "Tolerance Spreading"

It is noteworthy that the immune response against nuclear autoantigens is inter-connected by cross-reactive recognition at the B cell level (38, 39, 42), and importantly at the Th cell level (61, 101, 102, 105, 109). Thus the same lupus Th clone can help either a B cell specific for nucleosomes, or a B cell specific for dsDNA, or for ssDNA, or histone, or HMG, because each B cell can take up and process the whole chromatin particle by recognizing its own specific epitope in the chromatin, and then present the Th clone's relevant histone peptide epitope derived from chromatin processing, and that results in linked intermolecular help (56, 60, 120). Therefore, suppressing the Th cells of lupus could block spreading of response to multiple epitopes in chromatin (**Figure 4**). This hypothesis of "Tolerance Spreading" was experimentally supported as described above showing that progression of established lupus nephritis in the lupus-prone mouse models can be delayed, diminishing proteinuria and prolonging life by administering the nucleosomal peptide epitopes singly in high dose IV or low dose SC in tolerogenic regimens (80, 108, 109).

Indeed, the production of a variety of pathogenic autoantibodies to nuclear autoantigens was inhibited by tolerance therapy with any one of the epitopes. Due to promiscuous recognition described above, multiple autoimmune T cells with different TCRs can respond to the same peptide from a nucleosomal histone, and on the other hand, an individual autoimmune T cell can recognize multiple nucleosome-derived peptides that are distinct in sequence (61, 105). Therefore, when injected in a soluble tolerance-inducing form, in the absence of adjuvants, even one peptide epitope can tolerize autoimmune Th cells of diverse specificity for chromatin-derived autoantigens and conversely suppressing Th cells with specificty for one nuclear autoantigenic epitope deprives help for multiple autoimmune B cells of lupus. Thus, tolerance induced by any one of the dominant peptide epitopes can suppress autoimmune response to other nucleosome-derived pathogenic epitopes ("*Tolerance Spreading*"). Indeed, such "cross-reactive" suppression directed at the broad spectrum of pathogenic autoimmune response is more desirable in lupus therapy rather than pinpoint precision for antigen-specificity, which is the goal of some studies using modern techniques (121). Furthermore, the peptide epitopes are very effective in tolerance induction because, they are simultaneously recognized by autoimmune T and B cells, and they may inhibit autoimmune B cells and DC directly in lupus (80, 101, 108, 109).

Despite "Tolerance Spreading to other lupus autoepitopes", the histone peptide therapy resulted in autoantigen-specific regulation because, pathogenic autoimmune responses in lupus-prone subjects were preferentially and selectively downregulated, without any suppression of immune responses to foreign antigens, as detailed in these References (80, 108, 109). Moreover, ability of the treated animals to survive environmental microbes/pathogens appeared to be intact and robust as compared to untreated controls and non-autoimmune "normal" mice housed in the same "dirty" mouse facilities (80, 108, 109). Finally, experiments showed that the Treg cells generated by the peptide epitope therapy suppressed T cell response and T cell helper activity specifically directed to the peptide autoepitopes, but not for a foreign antigen, such as Hen Egg Lysozyme (80, 108).

Summary of Lessons Derived From Above Studies in Lupus-Prone Mice and Comparisons With Findings From Contemporary Literature

As described above, the nucleosomal histone peptide epitopes when administered in nanomolar doses (1μg) subcutaneously (S.C.) every 2 weeks or even every month to lupus-prone mice, are effective in delaying or even preventing nephritis. This dose is lower by almost 1000 fold compared to some other peptides being tried as therapeutic agents. Furthermore, a major histone peptide epitope, administered singly, can suppress lupus disease also *via* nasal tolerance (122, 123). The peptide epitopes are a constituent of nucleosome, a highly conserved, ubiquitous self-antigen produced during ongoing apoptosis in generative lymphoid organs and recognized by developing cells of the immune system bearing appropriate receptors. Therefore, anaphylactic reactions were not observed with these self-peptides when administered in close to 1000 lupus-prone mice for various studies. The histone peptide epitopes, administered S.C. in a very low doses, generate Treg cells that suppress by producing minute amounts of TGFβ that act in close range cell to cell interaction, rather than causing Th2 deviation with consequent allergic reactions seen in the case of therapy of other autoimmune diseases, such as, EAE/MS and diabetes in NOD mice using other peptides. The histone peptide epitopes induce stable Treg that are autoepitope-specific and cross-reactive autoantigen-directed Treg cells by simultaneously decreasing IL-6 and increasing TGF-β production by DCs, which consequently caused Smad-3 phosphorylation in the peptide epitope-specific auto-immune CD4+ Th cells, and the peptide tolerance therapy is effective even at an age when they manifest clinically active disease. A single dominant epitope such as $H4_{71\text{-}94}$, is capable of inhibiting the diverse autoimmune process in lupus, because, potent and durable regulatory T cells (Treg) are generated by low-dose tolerance therapy mediating "tolerance spreading". Both sets of regulatory T cells act *via* TGFβ in close range, and suppress autoimmune Th and B cells and other autoantigen presenting cells.

Although the phenomenon of low-dose tolerance was well known (124), most work since then have dealt with tolerance induction to foreign antigens in non-autoimmune subjects. However, the histone peptide induced low-dose tolerance was

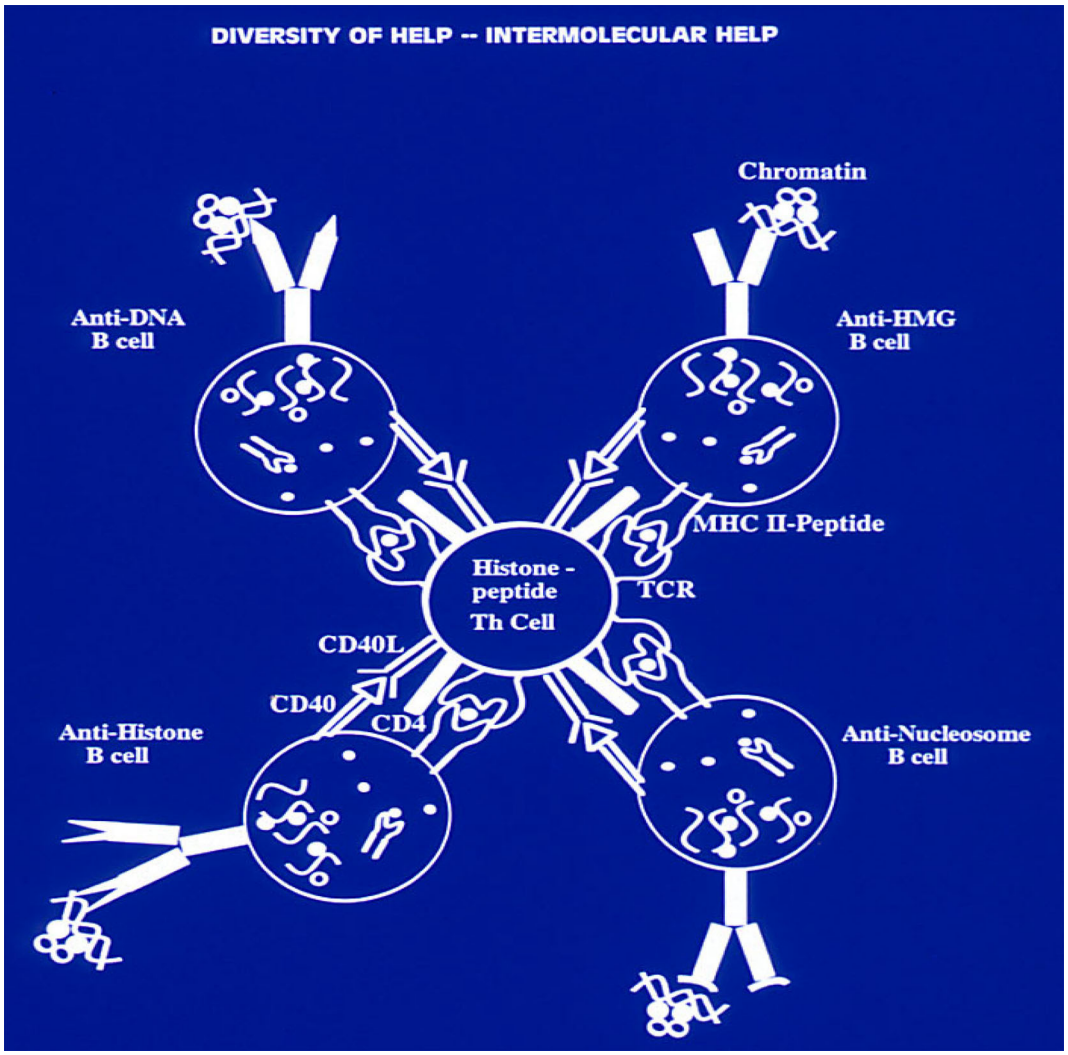


FIGURE 4 | Production of a variety of anti-nuclear autoantibodies by inter-molecular T-cell help in SLE. A lupus Th cell with specificity for an individual nucleosomal histone peptide can help either a B cell specific for nucleosomes, or a B cell specific for dsDNA, or for ssDNA, or histone, or HMG, because each B cell can take up and process the whole chromatin particle by recognizing its own specific epitope in the chromatin, and then present to the Th clone its relevant histone peptide epitope derived from chromatin processing, which results in "inter-molecular help" This principle of linked "inter-molecular help" for a variety of B-cell epitopes in the complex chromatin particle would also apply to other Th cells of lupus which induce other pathogenic autoantibodies; and forms the basis for "Tolerance-Spreading" as described in the text. Modified from Ref (109, 120). (Originally published in *The Journal of Immunology*. Kaliyaperumal A, Michaels MA, Datta SK. Antigen-specific therapy of murine lupus nephritis using nucleosomal peptides: Tolerance spreading impairs pathogenic function of autoimmune T and B cells. *J Immunol* (1999) 162:5775-83. Copyright © [1999] The American Association of Immunologists, Inc.).

achieved in subjects with spontaneous SLE, whose immune system is already primed for autoimmune response against ubiquitous nucleosomal self-epitopes. The Autoantigen-specific and cross-reactive autoantigen-directed Treg cells generated by the peptide epitope therapy was effective even in the presence of complex lupus abnormalities, such as hyperactivity of lupus T and B cells and DC; particularly IFN-α producing pDC.

Unlike the peptide epitopes in low doses, whole (intact) histones worsen lupus (60) probably by binding to DNA or reciprocally charged molecules *in vivo* to make complex nucleosome like particle structures. Moreover, processing by APC of intact histones generates altered epitopes by post-translational modifications, such as acetylation or citrulination (125). Therefore, intact whole (complete) histones should not be used for tolerance induction.

Another group induced Treg cells by continuous infusion of a model laboratory antigen using hemagglutinin (HA)-specific TCR–Tg mouse system, and they also targeted the peptide to a surface receptor DEC-205 on DC, and administered considerable quantities of TGFβ *in vivo* (126). However, those approaches for therapy of diabetes were found to be deleterious (127). Moreover, *in vivo* administration of TGF–β as a drug in the presence of high IL–6 levels in lupus could induce pathogenic Th17 cells and T_{FH} cells, instead of generating Treg cells (128, 129).

RECENT STUDIES IN HUMAN LUPUS RELEVANT TO THE PEPTIDE EPITOPES

Remarkably, pathogenic anti-DNA autoantibody inducing Th cells in human lupus recognized the same immunodominant histone peptide autoepitopes identified in murine lupus (61, 102), and those T cells in lupus patient's PBMC respond by producing IFNγ. To reiterate, IFNγ-dependent IgG autoantibody subclasses cause lupus nephritis by fixing C' and binding to inflammatory Fcγ receptors in pathogenic cells (39, 130). Furthermore, the peptide autoepitopes for Th cells of human lupus have the property of promiscuous HLA-DR binding, and as in lupus-prone mice, they are located in the native nucleosome at sites that contact with DNA, and they reside in histone regions that are also targeted by lupus B-cells (autoantibodies), thus being protected during antigen processing. Therefore, these immunodominant epitopes could probably be used as "universal" tolerogens in lupus patients despite their diversity of HLA alleles. The pathogenic role of nucleosome epitope-specific Th cells in human lupus have been confirmed by other laboratories (131, 132). Similar principles apply to other autoantigens in lupus, such as, Sm, RNP (133), but this review is focused on anti-DNA response whose pathogenic role in human lupus nephritis is well characterized. Remarkably, very recent approaches using latest technology to identify immunodominant epitopes for influenza hemagglutinin-specific memory T cells (134), showed results that are similar in outcome to the histone peptide approach performed two decades ago to identify the recurrent epitopes for pathogenic anti-DNA inducing memory T cells of lupus (61, 102).

(a). Publication Title: "Regulatory T Cell (Treg) Subsets Return in Patients With Refractory Lupus Following Stem Cell Transplantation and TGF-β Producing CD8+ Regulatory Treg Cells ($CD8^{TGF\text{-}\beta}$ Treg) Are Associated With Immunologic Remission of Lupus"

Unexpectedly, prolonged remission achieved by patients with refractory lupus after autologous hematopoietic stem cell transplantation (HSCT) have a different mechanistic basis than "clinical remission" in conventional drug-treated patients, who do not achieve true immunologic remission, although they have a Systemic Lupus Disease Activity Index (SLEDAI) of 0–2 (80% of the drug induced remission patients were at zero level). In patients with stem cell transplant induced remission, $CD4+CD25^{high}$FoxP3+ Treg, and CD8+FoxP3+ Treg cells are generated, accompanied by almost total suppression of pathogenic T cells that respond to the histone peptide autoepitopes (135).

Detailed experiments in the above ref (135)., demonstrated that the post-transplant CD8 Treg cells suppressive activity was nucleosomal histone peptide-specific, as well as nonspecific, but directed to cross-reactive autoreactive and activated T cells. Both types of Treg cell's suppressive activity was mainly TGF-β-dependent, but independent of cell-cell contact. The post-transplant patients' CD8 Treg cells were stably FoxP3+ and they expressed markedly increased levels of CTLA-4, CD103, PD-1, PD-L1 and LAP, when compared to CD8 T cells from the same patients before undergoing transplantation. By contrast, the pre-transplant lupus patient's CD8 T cells have cell-contact dependent helper activity for autoantibody production. The CD8 Treg found only in post-transplant patients are considerably more potent in suppressive activity compared to the $CD4+CD25^{high}$ Treg cells that appear during clinical "remission" in lupus patients treated by conventional drugs, in whom autoimmune response of CD4 T cells to nucleosome-derived autoepitopes persists even during "clinical remission" (SLEDAI of zero). Therefore, autologous HSCT leads to the generation of a newly differentiated population of $LAP^{high}CD103^{high}$ $CD8^{TGF\text{-}}\beta$ Treg cells that maintain the lupus patients in "*true immunological* remission", unlike patients with conventional drug therapy. Remarkably, very similar, highly potent CD8 Treg cells are also generated by low-dose nucleosomal peptide tolerance therapy that can prevent or treat lupus disease in mouse models of spontaneous SLE, as described above.

As stated, autoantibodies in lupus that belong to IFN-γ (Th1) dependent IgG subclasses fix complement and bind to activating FcγR on inflammatory cells to mediate pathogenicity. A CD4 T cell population in untreated lupus patients PBMC produces IFN-γ in response to histone peptide autoepitopes, and this autoimmune IFN-γ production response was almost completely suppressed in fresh PBMC from lupus patients in remission post-transplant. Removal of CD8 T cells (total) from the PBMC of post-transplant patients in remission, restored the IFN-γ response of CD4 T cells to nucleosomes and histone epitopes, much more strongly than removal of $CD4+CD25^{high}$ cell subset enriched for Treg. Therefore, the latter subset probably cannot restore immunologic remission in conventionally treated lupus patients although they are increased in such patients after "clinical remission" (SLEDAI of 0–2).

The Post-transplant CD8 T cells suppressed by secreting mainly TGF-β and they expressed high levels of TGF-β latency-associated peptide (LAP), but they produced IL-10 to a much lesser extent; which is desirable because IL-10, by causing expansion of autoimmune B cells, is deleterious in lupus (136).

Significance of the Above Studies in Lupus Patients Transplanted With Autologous Stem Cells and Contemporary Relevant Studies by Others

The return of potent $CD8^{TGF}$–β Treg cells after HSCT in refractory lupus patients, or after nucleosomal peptide epitope tolerance therapy in lupus-prone mice is an important biomarker for a state of true Immunologic Remission. These CD8+CD103+ FoxP3+ TGFβ producing Treg are highly effective in controlling lupus, as shown in autologous stem cell transplant patients in remission above; and after corticosteroid pulse therapy induced remission in patients with lupus nephritis (Tsai YG et al. Plos One 2014, 9:e81344); as well as in murine models of lupus (108, 137, 138), and graft-versus-host lupus (138–140). And the above category of CD8+FoxP3+ TGFβ producing Treg that are highly effective in controlling lupus disease, are quite different from

another variety of CD8+ Treg that are FoxP3-negative, cytotoxic and contact-dependent, and with varying surface phenotypes found in organ-specific autoimmune diseases (141–143). Those FoxP3$^-$Ly49+(CD158e+ in humans)CD122^{hi}Helios+CXCR5+ CD8 Treg cells were decreased as a percentage of total CD8 T cell population in lupus (144, 145), but such changes in proportion could be due to many reasons that cause shifts in various CD8 T cell subsets in lupus (146). Therefore, no cause and effect relationship of the latter CD8 Treg with spontaneous lupus disease in humans has been established yet. Anyway, target organ pathology in lupus nephritis, is inhibited by the TGFβ producing CD4$^+$FoxP3$^+$ Treg and CD8$^+$FoxP3$^+$ Treg cells induced by histone peptide epitope tolerance therapy (80, 108), or by targeted nanoparticle therapy (140) induced CD8+ Treg, which are quite different from the cytotoxic CD8 Treg (144, 145). Indeed, CD8+CD103+FoxP3+ TGFβ producing Treg cells, which maintain lupus patients in long term immunological remission after autologous bone marrow transplantation (135), or that induced in lupus patients' PBMC by the histone peptide epitopes *in vitro* (147), have their highly effective suppressor counterparts in several models of autoimmune diseases including lupus (148–151). The role of locally active tissue-resident TGFβ producing Treg cells migrating into the kidney and its lymph nodes to suppress lupus nephritis pathogenesis, like those induced by histone peptide epitope therapy, has been recently demonstrated in other Treg inducing systems (114–116, 152).

(b). Publication Title: "Major Pathogenic Steps in *Human* Lupus Can Be Effectively Suppressed by Nucleosomal Histone Peptide Epitope-Induced Regulatory Immunity"

As low-dose tolerance induced by the histone peptide epitopes effectively inhibited lupus disease in mouse models, the effect of the epitopes on lupus patients' PBMC cultures was tested *in vitro*. As discussed above, the major Peptide Autoepitopes for nucleosome-specific T Cells of human lupus were identical in sequence to the peptide autoepitopes for pathogenic T cells of lupus-prone mice (61, 102), and they shared similar properties of promiscuous MHC class II binding and being B cell autoantibody epitopes as well. Thus the peptide epitopes could be effective tolerogens for inhibiting both autoimmune T and B cell populations in lupus patients with diverse HLA alleles (61, 102, 105, 147).

Indeed, in PBMC cultures from inactive lupus patients and healthy subjects, addition of the histone peptide epitopes induced CD4$^+$CD25^{high}FoxP3$^+$ or CD4$^+$CD45RA$^+$FoxP3^{low} Treg cells, as well as CD8$^+$CD25$^+$FoxP3$^+$ Treg cells with stable FoxP3 expression and suppressive activity (147). In the case of PBMC from patients with active lupus, dexamethasone or hydroxychloroquine were additionally needed for Treg-induction by the peptide epitopes in cultures. The peptide-induced regulatory T cells in lupus PBMC depended on TGFβ/ALK-5/pSmad 2/3 signaling, and TGF-β precursor LAP was expressed by those Treg cells, indicating that TGFβ production was responsible for their suppressive activity, and a positive feedback mechanism as well. The peptide epitope-induced Treg cells also inhibited type I IFN related gene expression in lupus PBMC. Expression of major members of Type I IFN genes themselves, as well as type I IFN *induced* genes (ISG) were markedly reduced by histone peptide epitopes in TLR9-stimulated PBMC of lupus patients. As stated above, pDCs in lupus are the main producers of Type I IFN upon stimulation by nuclear autoantigens complexed with anti-nuclear autoantibodies (7, 153, 154), and in lupus-prone mice, histone peptide epitopes act on pDC rendering them to become tolerogenic (80). Secondly, expression of 13 ISG genes, which have been reported to be upregulated in patients with active lupus (153, 155, 156) were also inhibited by the peptide epitopes. Moreover, the histone peptide Th cell epitopes, which were also shared by autoantibody producing B cell epitopes in lupus, could inhibit production of pathogenic autoantibodies by PBMC from *active* lupus patients as potently as an anti-IL6 antibody. Experimental details are in reference (147). Importantly, a mixture of the peptide epitopes (*cocktail)* was more effective in uniformly suppressing pathogenic activities in *Human* lupus PBMC cultures, as compared to single epitopes, because patients are heterogeneous in contrast to inbred lupus-prone mice. For example, suppression by a histone peptide cocktail #1 (C1), which is a mixture of H1$^{22\text{-}42}$, H3$^{115\text{-}135}$ and H4$^{16\text{-}39}$ at a concentration of 1.5 μM for each peptide or histone peptide epitope cocktail #2 (C2), which is a mixture of H1$^{22\text{-}42}$, H3$^{115\text{-}135}$ and H4$^{16\text{-}39}$ at a concentration of 4 μM of each peptide were very efficient in suppressing pathogenic autoantibody production and type I IFN related gene expression in lupus PBMC (147). Thus, low-dose histone peptide epitopes could durably inhibit pathogenic autoimmune response in human lupus by diverse pathways.

OVERALL CLINICAL SIGNIFICANCE AND SUMMARY

Generalized immunosuppression can control manifestations of active lupus, but despite their toxicity the drugs fail to achieve true immunological remission. Such drug therapies should be followed by autoantigen specific suppression of pathogenic autoimmune cells in lupus to prevent flares and continuing organ damage. In contrast to lupus patients, normal subjects have regulatory mechanisms including regulatory T cells that prevent abnormal pathogenic response to nuclear autoantigens from cells undergoing apoptosis routinely in the body (135, 147, 157).

The tolerogenic histone peptide epitopes have the potential for prophylactically repairing the functional deficiency of regulatory T cells in lupus (135, 147, 157, 158). The above studies in mouse models *in vivo*, and with lupus patient's cells *in vitro*, showed that the peptide autoepitopes have the ability to bring about durable regulatory mechanisms; probably because of desirable properties mentioned above, and summarized here. The histone peptide epitopes are derived from nucleosomes of apoptotic cells produced daily in the body, which are cleared

silently in the normal host without causing any immune response (17). Indeed large scale apoptosis occurs daily in generative organs, such as bone marrow and thymus and the products are used to "educate" the cells of developing immune system. The epitopes with their native sequences intact are called "unaltered peptide ligands (UPL)"; they are derived from nucleosomes of apoptotic cells that are naturally processed and displayed to developing lymphocytes during ontogeny (101, 107, 159, 160), and therefore, unlike artificially altered peptide ligands (APL), or post-translationally acetylated or citrullinated histone peptides, the unaltered histone peptide epitopes described here are not associated with anaphylactic/allergic reactions or worsening of lupus (80, 108, 109). In fact Treg cells are generated in the thymus, even in lupus-prone mice, in a natural response to the native unaltered histone peptide epitopes (107),. Only 1 μg (0.34 nanomolar) of the histone peptide epitope/s administered biweekly is effective in low-dose tolerance therapy of lupus-prone mice; that dosage would be around 0.2 to 2 mg range in humans with lupus. The histone peptides are rapidly absorbed systemically after S.C. injection, because they possess numerous charged residues making them highly soluble. As soon as they reach the lymphoid organs the peptides render APCs, especially pDC tolerogenic by inducing TGFβ and inhibiting IL-6, and consequently the peptide epitope presenting DC generate long-lasting Treg containing autoantigen-specific and cross-reactive autoantigen-directed Treg and Treg cells that suppress lupus (80, 108). Because the peptide epitopes operate by being taken up extremely rapidly by DC *in vivo* rendering them tolerogenic, short half life due to decay of the epitopes is not a problem. Moreover, the histone peptide therapy induced stable autoantigen-specific and cross-reactive autoantigen-directed regulatory or suppressive T cells generated *in vivo* are effective in suppressing disease upon transfer into lupus-prone mice (80, 108). Both MHC class II, and nested MHC class I binding determinants are present in the peptide epitope sequences so that they can genarate both CD4 Treg and CD8 Treg cells (80, 108). The epitopes are recognized by autoimmune T cells irrespective of the HLA type of lupus patients (102, 105, 135, 147, 159), similar to "universal epitopes" (104). Tolerance therapy with the histone peptide epitopes is effective even in mice with established lupus disease (80, 108, 109). The peptides can generate Treg in lupus patient's PBMC even in the presence of other conventional maintenance medicines such as hydroxychloroquine or corticosteroids (147). The peptide autoepitopes from histones induce "linked tolerance" to other nuclear antigen autoepitopes recognized by pathogenic T and B cell of lupus (cross-reactive, "tolerance spreading"), but not to foreign antigens or other organ-derived autoantigens. In addition to generation of Treg cells, the peptides also exert tolerogenic effect directly on pathogenic lupus B cells and DC (80, 101, 147); suppressing autoantibody production irrespective of the degree of Treg induction (135, 147). Regulatory mechanisms against abnormal autoimmune response to nuclear autoantigens in asymptomatic subjects could be enforced by the relatively innocuous tolerance therapy with histone peptides (147), which suggests that apparently healthy relatives or ANA positive subjects at risk for developing lupus as predicted by GWAS bio-markers, could be protected prophylactically with these peptide epitopes.

Thus the peptide epitope therapy might be most suitable for maintaining lupus patients in true immunological remission after clinical remission has been induced by more toxic immunosuppressive agents. To summarize, unlike pinpoint antigen-specific therapy suitable for straight-forward organ-specific autoimmune diseases, the histone peptide epitopes directly or indirectly (through Treg cells they induce) suppress Innate immune cells (DC), T cells and B cells involved in the pathogenic autoimmune response in the complex systemic autoimmune disease, Lupus.

The histone peptide epitopes could also be used to develop sensitive diagnostic and/or prognostic tools (peptide-MHC tetramers) or assays (intracellular cytokine response) for tracking pathogenic Th cells that may appear prior to manifestation of the disease and elevation of autoantibodies. Indeed, understanding mechanism/s for generation of unusual and potent CD8+ Treg cells by the peptide therapy will be of therapeutic value in a broad spectrum of immune mediated diseases, and Immunologic Monitoring with the peptide epitopes may serve as biomarkers for true immunologic remission (supplementing conventional measures of clinical remission, such as, SLEDAI SLAM, BILAG).

FUTURE –PERSPECTIVE, PROBLEMS THAT MAY ARISE, AND POSSIBLE ANSWERS

Early phase clinical trials have shown promising outcome with autoantigen peptide therapy for inducing antigen-specific tolerance in several autoimmune diseases, such as Multiple Sclerosis and Type 1 Diabetes (161–167). These results are encouraging for clinical trials with histone peptides for lupus in the near future, but several distinct features of this lupus therapy need to be addressed to reach that goal. Unlike pinpoint antigen-specific therapy suitable for straight-forward organ-specific autoimmune diseases, the histone peptide epitopes have unique tolerogenic properties with broad autoreactivity-specific inhibitory effect. By rendering Innate immune cells (DC) tolerogenic, the histone peptides induce Treg cells that suppress T and B cell populations which are both antigen-specifically and cross-reactively involved in the pathogenic autoimmune response in the complex systemic autoimmune disease, Lupus:

a) Low-Dose IL-2 and Corticosteroid Supplementation

Multiple laboratories have shown that histone peptide epitope/s or other peptide epitopes administered without IL-2 injection, can induce generation of effective Treg *in vivo*, which inhibit disease in various lupus-prone mice (80, 82–85, 108, 122, 168). Although lupus T cells are deficient in IL-2 production (169, 170), that situation is relative not absolute, as lupus patients do not succumb to recurrent infections found in IL-2 knockout

Immunodeficiency. A possibility in the case of these peptide epitopes is that in low doses, they could transiently activate autoreactive T cells, which could then provide small amounts of IL-2 for generating the regulatory T cells. Indeed this proposed mechanism (171), has actually been demonstrated by a similar situation occurring in the thymus where IL-2 is produced by a small population of self-reactive CD4 single positive (CD4SP) thymocytes, which then stimulates Treg precursor cells to differentiate (172). Those regulatory T cells induced *in vivo* may then be sustained also by other signals such as from ICOS, or TNFR2 (Tseng WY et al. Proc Natnl Acad Sci USA 2019, 116:21666-21672) (173). Still in view of the benefits of low dose IL-2 therapy in all autoimmune diseases, whether deficient in IL-2 or not (129, 169, 170, 174–177); adjunct therapy with low-dose IL-2 will be beneficial in the peptide-epitope therapy of lupus, as stated in the theme of this Topic. Moreover, Low dose IL-2 and corticosteroids in maintenance dose, actually were necessary for the peptide epitopes' induction of Treg cells in ACTIVE lupus patients' PBMC *in vitro* (147). Indeed, corticosteroids themselves induce Treg cells by various mechanisms to some extent (Tsai YG et al. Plos One 2014, 9:e81344) (178, 179), and thus could potentiate the autoantigen-specific and cross-reactive autoantigen-directed Treg response by the peptide epitopes (147).

b) Durable Treg Induction in the Midst of Inflammation; and Intrinsic Tolerogenic Properties of the Histone Peptide Epitopes

How can durable immunoregulatory mechanisms be established in the inflammatory environment of lupus? In lupus patients, tolerance therapy with histone peptide epitope would be optimal after inflammatory burden is reduced by drugs. Nevertheless, in animal models, the peptides alone are effective in ameliorating established lupus nephritis (80, 108, 109). The regulatory T cells are more stable in inflammatory environment because they were induced by the peptide epitopes *in vivo*, in contrast to Treg cells induced/expanded *in vitro*. Furthermore, dexamethasone or hydroxychloroquine in maintenance doses actually supported Treg-induction by the peptides in lupus patients' PBMC cultures, indicating that drugs that counter the increased activity of IRF5 and TLR pathways in lupus APC would be of added benefit (147). The histone peptide epitopes also can directly regulate autoimmune B cells and DC in lupus, in addition to generating Treg cells (80, 101, 109); and indeed the peptides could suppress autoantibody production to baseline levels in lupus patient's PBMC even before significantly increasing Treg cell numbers in culture (147). The select histone peptide epitopes, which are tolerogenic, can directly reduce IL-6 and increase TGFβ production by DC (80), a situation which renders the DC not only be able to induce Treg, but also become susceptible to suppression by Treg (180). This property of inducing TGFβ production and simultaneously decreasing IL-6 production by DC, especially pDC, in turn induces TGF-β signal (Smad-3 phosphorylation) in target auto-immune CD4+ T cells converting them to stable Treg cells; a property highly beneficial for lupus therapy (80, 147), also because T_{FH} cell differentiation is inhibited in germinal centers under such conditions (128).

Since apoptotic cells have immunosuppressive properties (17, 181); the unaltered histone peptide epitopes derived from apoptotic platform may have intrinsic tolerogenic property (80, 147). In very low doses, without immunostimulatory adjuvants, the histone peptide epitopes could possibly activate latent TGFβ, by inducing expression of the integrin αvβ8 in resting pDC, as shown in other systems (182).

c) Peptide Delivery

Treg cells require continued antigen-specific stimulation from DC to maintain lineage stability and high affinity regulatory cell function (183, 184). In lupus-prone mice, regulatory T cells induced by the peptide epitopes are detectable up to six weeks after S.C. injection. Subcutaneous injection route works for the highly soluble and charged histone peptide epitopes which are very rapidly absorbed systemically (80). In humans many protein drugs, and mAb biologics, insulin, IVIg etc., are administered S.C. without causing local/systemic inflammatory response.

However, despite the promising beneficial effects in animal models of established lupus disease, there is always the possibility of adverse autoreactive response to the peptide epitopes in patients with lupus, although they might be selected at the earliest, pre-clinical stage of disease. Another issue is that peptide epitope cocktails in low doses were more effective than a single peptide epitope in suppressing lupus manifestations in human lupus PBMC, but cocktails may be more immunogenic when injected in the skin (147).

Therefore, peptide delivery should be considered in fail-safe tolerogenic vehicles, such as Nanoparticles (NP), which are described in detail by experts in this field in other articles as part of this research topic. Just as a brief synopsis, the peptide epitopes may be delivered within the nanoparticles, or administered around the same time, but separately from tolerogenic nanoparticles (185). There are many issues in choosing the right nanoparticles for such therapy, specifically for lupus; for instance, liposome derived NP can activate complement, and rapamycin containing NP may interfere with initial Treg generation (186, 187), although rapamycin is effective in maintaining Treg, once they are induced (188). It is noteworthy that injected nanoparticles might be nonspecifically immunosuppressive, like silica particles, by overloading the immune system's APCs, which phagocytose and engorge themselves with any foreign particles (189, 190). Therefore, targeted nanoparticles designed to be directed against potentially autoreactive T cells are much more promising (140, 191), as addressed by articles from experts in this research topic.

Finally, emerging studies on epigenetic or metabolic mechanisms for Treg cell stability (192–194), and correcting other abnormalities in lupus T cells, such as, metabolic (12, 13), could be potentiated by utilizing the benefits of peptide epitope therapy, in the near future.

AUTHOR CONTRIBUTIONS

SD wrote and edited this review, and is accountable for the content of the work.

FUNDING

This is a review of published work. Details of funding are in those publications cited in this review, and the work by the author's group at that time had been supported partially by funding from National Institutes of Health (NIAID for 43 years, and NIAMS for 30 years), and Alliance for Lupus Research (TIL grant for 6 years).

ACKNOWLEDGMENTS

The studies reviewed (and cited) in this paper are from collaborative work with many talented scientists, who were mainly at Northwestern University Feinberg School of Medicine at the time the studies were done, especially: Arunan Kaliyaperumal, Hee-Kap Kang, Li Zhang, Chandra Mohan, Sharlene Adams, Sumi Rajagopalan (Shivakumar), Rosalind Ramsey Goldman, and Richard Burt.

REFERENCES

1. Alarcón-Riquelme ME, Ziegler JT, Molineros J, Howard TD, Moreno-Estrada A, Sánchez-Rodríguez E, et al. Genome-Wide Association Study in an Amerindian Ancestry Population Reveals Novel Systemic Lupus Erythematosus Risk Loci and the Role of European Admixture. *Arthritis Rheumatol (Hoboken NJ)* (2016) 68(4):932–43. doi: 10.1002/art.39504
2. Barcellos LF, May SL, Ramsay PP, Quach HL, Lane JA, Nititham J, et al. High-density SNP screening of the major histocompatibility complex in systemic lupus erythematosus demonstrates strong evidence for independent susceptibility regions. *PloS Genet* (2009) 5(10):e1000696. doi: 10.1371/journal.pgen.1000696
3. Bentham J, Morris DL, Graham DSC, Pinder CL, Tombleson P, Behrens TW, et al. Genetic association analyses implicate aberrant regulation of innate and adaptive immunity genes in the pathogenesis of systemic lupus erythematosus. *Nat Genet* (2015) 47(12):1457–64. doi: 10.1038/ng.3434
4. Martin JE, Assassi S, Diaz-Gallo LM, Broen JC, Simeon CP, Castellvi I, et al. A systemic sclerosis and systemic lupus erythematosus pan-meta-GWAS reveals new shared susceptibility loci. *Hum Mol Genet* (2013) 22(19):4021–9. doi: 10.1093/hmg/ddt248
5. Krausgruber T, Blazek K, Smallie T, Alzabin S, Lockstone H, Sahgal N, et al. IRF5 promotes inflammatory macrophage polarization and TH1-TH17 responses. *Nat Immunol* (2011) 12(3):231–8. doi: 10.1038/ni.1990
6. Stone RC, Feng D, Deng J, Singh S, Yang L, Fitzgerald-Bocarsly P, et al. Interferon regulatory factor 5 activation in monocytes of systemic lupus erythematosus patients is triggered by circulating autoantigens independent of type I interferons. *Arthritis Rheumatol* (2012) 64(3):788–98. doi: 10.1002/art.33395
7. Niewold TB, Kelly JA, Flesch MH, Espinoza LR, Harley JB, Crow MK. Association of the IRF5 risk haplotype with high serum interferon-alpha activity in systemic lupus erythematosus patients. *Arthritis Rheumatol* (2008) 58(8):2481–7. doi: 10.1002/art.23613
8. Yan J, Hedl M, Abraham C. Myeloid Cell-Intrinsic IRF5 Promotes T Cell Responses through Multiple Distinct Checkpoints In Vivo, and IRF5 Immune-Mediated Disease Risk Variants Modulate These Myeloid Cell Functions. *J Immunol (Baltimore Md 1950)* (2020) 205(4):1024–38. doi: 10.4049/jimmunol.1900743
9. Wang Y, Chen S, Chen J, Xie X, Gao S, Zhang C, et al. Germline genetic patterns underlying familial rheumatoid arthritis, systemic lupus erythematosus and primary Sjögren's syndrome highlight T cell-initiated autoimmunity. *Ann Rheumatic Dis* (2020) 79(2):268–75. doi: 10.1136/annrheumdis-2019-215533
10. Kamitaki N, Sekar A, Handsaker RE, de Rivera H, Tooley K, Morris DL, et al. Complement genes contribute sex-biased vulnerability in diverse disorders. *Nature* (2020) 582(7813):577–81. doi: 10.1038/s41586-020-2277-x
11. Desai-Mehta A, Lu L, Ramsey-Goldman R, Datta SK. Hyperexpression of CD40 ligand by B and T cells in human lupus and its role in pathogenic autoantibody production. *J Clin Invest* (1996) 97:2063–73. doi: 10.1172/JCI118643
12. Yin Y, Choi SC, Xu Z, Perry DJ, Seay H, Croker BP, et al. Normalization of CD4+ T cell metabolism reverses lupus. *Sci Trans Med* (2015) 7(274):274ra18. doi: 10.1126/scitranslmed.aaa0835
13. Li W, Elshikha AS, Cornaby C, Teng X, Abboud G, Brown J, et al. T cells expressing the lupus susceptibility allele Pbx1d enhance autoimmunity and atherosclerosis in dyslipidemic mice. *JCI Insight* (2020) 5(11):e138274. doi: 10.1172/jci.insight.138274
14. Giles JR, Neves AT, Marshak-Rothstein A, Shlomchik MJ. Autoreactive helper T cells alleviate the need for intrinsic TLR signaling in autoreactive B cell activation. *JCI Insight* (2017) 2(4):e90870. doi: 10.1172/jci.insight.90870
15. Steinman RM, Turley S, Mellman I, Inaba K. The induction of tolerance by dendritic cells that have captured apoptotic cells. *J Exp Med* (2000) 191(3):411–6. doi: 10.1084/jem.191.3.411
16. Steinman RM, Hawiger D, Nussenzweig MC. Tolerogenic dendritic cells. *Annu Rev Immunol* (2003) 21:685–711. doi: 10.1146/annurev.immunol.21.120601.141040
17. Voll RE, Herrmann M, Roth EA, Stach C, Kalden JR, Girkontaite I. Immunosuppressive effects of apoptotic cells. *Nature* (1997) 390:350–1. doi: 10.1038/37022
18. Skoberne M, Beignon AS, Larsson M, Bhardwaj N. Apoptotic cells at the crossroads of tolerance and immunity. *Curr Topics Microbiol Immunol* (2005) 289:259–92. doi: 10.1007/3-540-27320-4_12
19. Leadbetter EA, Rifkin IR, Hohlbaum AM, Beaudette BC, Shlomchik MJ, Marshak-Rothstein A. Chromatin-IgG complexes activate B cells by dual engagement of IgM and Toll-like receptors. *Nature* (2002) 416:595–8. doi: 10.1038/416603a
20. Means TK, Latz E, Hayashi F, Murali MR, Golenbock DT, Luster AD. Human lupus autoantibody-DNA complexes activate DCs through cooperation of CD32 and TLR9. *J Clin Invest* (2005) 115(2):407–17. doi: 10.1172/JCI23025
21. Chaturvedi A, Dorward D, Pierce SK. The B cell receptor governs the subcellular location of Toll-like receptor 9 leading to hyperresponses to DNA-containing antigens. *Immunity* (2008) 28(6):799–809. doi: 10.1016/j.immuni.2008.03.019
22. Souyris M, Cenac C, Azar P, Daviaud D, Canivet A, Grunenwald S, et al. TLR7 escapes X chromosome inactivation in immune cells. *Sci Immunol* (2018) 3(19):eaap8855. doi: 10.1126/sciimmunol.aap8855
23. Subramanian S, Tus K, Li QZ, Wang A, Tian XH, Zhou J, et al. A Tlr7 translocation accelerates systemic autoimmunity in murine lupus. *Proc Natnl Acad Sci USA* (2006) 103(26):9970–5. doi: 10.1073/pnas.0603912103
24. Deane JA, Pisitkun P, Barrett RS, Feigenbaum L, Town T, Ward JM, et al. Control of toll-like receptor 7 expression is essential to restrict autoimmunity and dendritic cell proliferation. *Immunity* (2007) 27(5):801–10. doi: 10.1016/j.immuni.2007.09.009
25. Heinz LX, Lee J, Kapoor U, Kartnig F, Sedlyarov V, Papakostas K, et al. TASL is the SLC15A4-associated adaptor for IRF5 activation by TLR7-9. *Nature* (2020) 581(7808):316–22. doi: 10.1038/s41586-020-2282-0
26. Pisetsky DS. The role of TASL in the pathogenesis of SLE: X marks the spot. *Ann Rheumatic Dis* (2021) 80(1):6–7. doi: 10.1136/annrheumdis-2020-218643
27. Bocharnikov AV, Keegan J, Wacleche VS, Cao Y, Fonseka CY, Wang G, et al. PD-1hiCXCR5- T peripheral helper cells promote B cell responses in lupus via MAF and IL-21. *JCI Insight* (2019) 4(20):e130062. doi: 10.1172/jci.insight.130062
28. Phalke S, Marrack P. Age (autoimmunity) associated B cells (ABCs) and their relatives. *Curr Opin Immunol* (2018) 55:75–80. doi: 10.1016/j.coi.2018.09.007
29. Kang HK, Chiang MY, Ecklund D, Zhang L, Ramsey-Goldman R, Datta SK. Megakaryocyte progenitors are the main APCs inducing Th17 response to lupus autoantigens and foreign antigens. *J Immunol (Baltimore Md 1950)* (2012) 188(12):5970–80. doi: 10.4049/jimmunol.1200452
30. Finkielsztein A, Schlinker AC, Zhang L, Miller WM, Datta SK. Human megakaryocyte progenitors derived from hematopoietic stem cells of normal individuals are MHC class II-expressing professional APC that enhance

Th17 and Th1/Th17 responses. *Immunol Lett* (2015) 163(1):84–95. doi: 10.1016/j.imlet.2014.11.013
31. Chen CI, Zhang L, Datta SK. Hematopoietic stem and multipotent progenitor cells produce IL-17, IL-21 and other cytokines in response to TLR signals associated with late apoptotic products and augment memory Th17 and Tc17 cells in the bone marrow of normal and lupus mice. *Clin Immunol (Orlando Fla)* (2016) 162:9–26. doi: 10.1016/j.clim.2015.10.007
32. Zufferey A, Speck ER, Machlus KR, Aslam R, Guo L, McVey MJ, et al. Mature murine megakaryocytes present antigen-MHC class I molecules to T cells and transfer them to platelets. *Blood Adv* (2017) 1(20):1773–85. doi: 10.1182/bloodadvances.2017007021
33. Rahman A, Isenberg DA. Systemic lupus erythematosus. *N Engl J Med* (2008) 358(9):929–39. doi: 10.1056/NEJMra071297
34. Liu Z, Davidson A. Taming lupus-a new understanding of pathogenesis is leading to clinical advances. *Nat Med* (2012) 18(6):871–82. doi: 10.1038/nm.2752
35. Rothfield NF, Stollar BD. The relation of immunoglobulin class, pattern of anti-nuclear antibody, and complement-fixing antibodies to DNA in sera from patients with systemic lupus erythematosus. *J Clin Invest* (1967) 46:1785–94. doi: 10.1172/JCI105669
36. Koffler D, Carr RI, Agnello V, Thoburn R, Kunkel HG. Antibodies to polynucleotides in human sera; antigenic specificity and relation to disease. *J Exp Med* (1971) 134:294–312. doi: 10.1084/jem.134.1.294
37. Ebling F, Hahn BH. Restricted subpopulations of DNA antibodies in kidneys of mice with systemic lupus: comparison of antibodies in serum and renal eluates. *Arthritis Rheumatol* (1980) 23:392–403. doi: 10.1002/art.1780230402
38. Gavalchin J, Nicklas J, Eastcott JW, Madaio MP, Stollar BD, Schwartz RS, et al. Lupus prone (SWR x NZB)F1 mice produce potentially nephritogenic autoantibodies inherited from the normal SWR parent. *J Immunol* (1985) 134:885–94.
39. Gavalchin J, Datta SK. The NZB x SWR model of lupus nephritis. II. Autoantibodies deposited in renal lesions show a restricted idiotypic diversity. *J Immunol* (1987) 138:138–48.
40. Vlahakos DV, Foster MH, Adams S, Katz M, Ucci AA, Barrett KJ, et al. Anti-DNA antibodies form immune deposits at distinct glomerular and vascular sites. *Kidney Internatnl* (1992) 41:1690–700. doi: 10.1038/ki.1992.242
41. Datta SK, Patel H, Berry D. Induction of a cationic shift in IgG anti-DNA autoantibodies. Role of T helper cells with classical and novel phenotypes in three murine models of lupus nephritis. *J Exp Med* (1987) 165:1252–68. doi: 10.1084/jem.165.5.1252
42. Gavalchin J, Seder RA, Datta SK. The NZB x SWR model of lupus nephritis. I. cross-reactive idiotypes of monoclonal anti-DNA antibodies in relation to antigenic specificity, charge and allotype. Identification of interconnected idiotype families inherited from the normal SWR and the autoimmune NZB parents. *J Immunol* (1987) 138:128–37.
43. O'Keefe TL, Bandyopadhyay S, Datta SK, Imanishi-Kari T. Variable region sequences of an idiotypically connected family of pathogenic anti-DNA autoantibodies. *J Immunol* (1990) 144:4275–83.
44. Radic MZ, Weigert M. Genetic and structural evidence for antigen selection of anti-DNA antibodies. *Annu Rev Immunol* (1994) 12:487–520. doi: 10.1146/annurev.iy.12.040194.002415
45. Shlomchik MJ, Mascelli M, Shan H, Radic MZ, Pisetsky D, Marshak-Rothstein A, et al. Anti-DNA antibodies from autoimmune mice arise by clonal expansion and somatic mutation. *J Exp Med* (1990) 171:265–92. doi: 10.1084/jem.171.1.265
46. Rennke HG, Cotran RS, Venkatachalam MA. Role of molecular charge in glomerular permeability. Tracer studies with cationized ferritin. *J Cell Biol* (1975) 67:638–46. doi: 10.1083/jcb.67.3.638
47. Kanwar YS, Farquhar MG. Anionic sites in glomerular basement membrane. In vivo and in vitro localization to the laminae rarae by cationic probes. *J Cell Biol* (1976) 81:137–53. doi: 10.1083/jcb.81.1.137
48. Kohro-Kawata J, Wang P, Kawata Y, Matsuzaki M, Nakamura K. Highly cationic anti-DNA antibodies in patients with lupus nephritis analyzed by two-dimensional electrophoresis and immunoblotting. *Electrophoresis* (1998) 19(8-9):1511–5. doi: 10.1002/elps.1150190849
49. Berden JH. Lupus nephritis. *Kidney Internatnl* (1997) 52:538–58. doi: 10.1038/ki.1997.365
50. van der Vlag J, Berden JH. Lupus nephritis: role of antinucleosome autoantibodies. *Semin Nephrol* (2011) 31(4):376–89. doi: 10.1016/j.semnephrol.2011.06.009
51. Olin AI, Mörgelin M, Truedsson L, Sturfelt G, Bengtsson AA. Pathogenic mechanisms in lupus nephritis: Nucleosomes bind aberrant laminin β1 with high affinity and colocalize in the electron-dense deposits. *Arthritis Rheumatol (Hoboken NJ)* (2014) 66(2):397–406. doi: 10.1002/art.38250
52. Kalaaji M, Mortensen E, Jørgensen L, Olsen R, Rekvig OP. Nephritogenic lupus antibodies recognize glomerular basement membrane-associated chromatin fragments released from apoptotic intraglomerular cells. *Am J Pathol* (2006) 168(6):1779–92. doi: 10.2353/ajpath.2006.051329
53. Sainis K, Datta SK. CD4+ T cell lines with selective patterns of autoreactivity as well as CD4-/CD8- T helper cell lines augment the production of idiotypes shared by pathogenic anti-DNA autoantibodies in the NZB x SWR model of lupus nephritis. *J Immunol* (1988) 140:2215–24.
54. Shivakumar S, Tsokos GC, Datta SK. T cell receptor alpha/beta expressing double negative ($CD4^-/CD8^-$) and $CD4^+$ T helper cells in humans augment the production of pathogenic anti-DNA autoantibodies associated with lupus nephritis. *J Immunol* (1989) 143:103–12.
55. Rajagopalan S, Zordan T, Tsokos GC, Datta SK. Pathogenic anti-DNA autoantibody inducing T helper cell lines from patients with active lupus nephritis: Isolation of CD4-/CD8- T helper cell lines that express the γδ T-cell receptor. *Proc Natl Acad Sci USA* (1990) 87:7020–4. doi: 10.1073/pnas.87.18.7020
56. Desai-Mehta A, Mao C, Rajagopalan S, Robinson T, Datta SK. Structure and specificity of T-cell receptors expressed by pathogenic anti-DNA autoantibody-inducing T cells in human lupus. *J Clin Invest* (1995) 95:531–41. doi: 10.1172/JCI117695
57. Adams S, Leblanc P, Datta SK. Junctional region sequences of T-cell receptor β-chain genes expressed by pathogenic anti-DNA autoantibody-inducing helper T cells from lupus mice: Possible selection by cationic autoantigens. *Proc Natl Acad Sci United States America* (1991) 88(24):11271–5. doi: 10.1073/pnas.88.24.11271
58. Mohan C, Shi Y, Laman JD, Datta SK. Interaction between CD40 and its ligand gp39 in the development of murine lupus nephritis. *J Immunol* (1995) 154:1470–80.
59. Mao C, Osman GE, Adams S, Datta SK. T cell receptor alpha-chain repertoire of pathogenic autoantibody-inducing T cells in lupus mice. *J Immunol* (1994) 152:1462–70.
60. Mohan C, Adams S, Stanik V, Datta SK. Nucleosome: A major immunogen for pathogenic autoantibody-inducing T cells of lupus. *J Exp Med* (1993) 177(5):1367–81. doi: 10.1084/jem.177.5.1367
61. Kaliyaperumal A, Mohan C, Wu W, Datta SK. Nucleosomal peptide epitopes for nephritis-inducing T helper cells of murine lupus. *J Exp Med* (1996) 183:2459–69. doi: 10.1084/jem.183.6.2459
62. Mitchison NA. T-cell-B-cell cooperation. *Nat Rev Immunol* (2004) 4(4):308–12. doi: 10.1038/nri1334
63. Datta SK, Schwartz RS. Genetics of expression of xenotropic virus and autoimmunity in NZB mice. *Nature* (1976) 263:412–5. doi: 10.1038/263412b0
64. Datta SK, McConahey PJ, Manny N, Theofilopoulos AN, Dixon FJ, Schwartz RS. Genetic studies of autoimmunity and retrovirus expression in crosses of NZB mice. II. The viral envelope glycoprotein gp 70. *J Exp Med* (1978) 147:872–81. doi: 10.1084/jem.147.3.872
65. Cook HT, Botto M. Mechanisms of Disease: the complement system and the pathogenesis of systemic lupus erythematosus. *Nat Clin Pract Rheumatol* (2006) 2(6):330–7. doi: 10.1038/ncprheum0191
66. Shao WH, Cohen PL. Disturbances of apoptotic cell clearance in systemic lupus erythematosus. *Arthritis Res Ther* (2011) 13(1):202. doi: 10.1186/ar3206
67. Rogers NJ, Lees MJ, Gabriel L, Maniati E, Rose SJ, Potter PK, et al. A defect in Marco expression contributes to systemic lupus erythematosus development via failure to clear apoptotic cells. *J Immunol (Baltimore Md 1950)* (2009) 182(4):1982–90. doi: 10.4049/jimmunol.0801320
68. Munoz LE, Lauber K, Schiller M, Manfredi AA, Herrmann M. The role of defective clearance of apoptotic cells in systemic autoimmunity. *Nat Rev Rheumatol* (2010) 6(5):280–9. doi: 10.1038/nrrheum.2010.46
69. Casciola-Rosen LA, Anhalt G, Rosen A. Autoantigens targeted in systemic lupus erythematosus are clustered in two populations of surface structures

on apoptotic keratinocytes. *J Exp Med* (1994) 179:1317–30. doi: 10.1084/jem.179.4.1317
70. Mevorach D, Mascarenhas JO, Gershov D, Elkon KB. Complement-dependent clearance of apoptotic cells by human macrophages. *J Exp Med* (1998) 188:2313–20. doi: 10.1084/jem.188.12.2313
71. Savill J, Fadok V. Corpse clearance defines the meaning of cell death. *Nature* (2000) 407(6805):784–8. doi: 10.1038/35037722
72. Poon IK, Lucas CD, Rossi AG, Ravichandran KS. Apoptotic cell clearance: basic biology and therapeutic potential. *Nat Rev Immunol* (2014) 14(3):166–80. doi: 10.1038/nri3607
73. Pisitkun P, Deane JA, Difilippantonio MJ, Tarasenko T, Satterthwaite AB, Bolland S. Autoreactive B cell responses to RNA-related antigens due to TLR7 gene duplication. *Sci (N Y NY)* (2006) 312(5780):1669–72. doi: 10.1126/science.1124978
74. Lartigue A, Colliou N, Calbo S, Francois A, Jacquot S, Arnoult C, et al. Critical role of TLR2 and TLR4 in autoantibody production and glomerulonephritis in lpr mutation-induced mouse lupus. *J Immunol (Baltimore Md 1950)* (2009) 183(10):6207–16. doi: 10.4049/jimmunol.0803219
75. Magna M, Pisetsky DS. The role of HMGB1 in the pathogenesis of inflammatory and autoimmune diseases. *Mol Med* (2014) 20:138–46. doi: 10.2119/molmed.2013.00164
76. Bianchi ME. HMGB1 loves company. *J Leukoc Biol* (2009) 86(3):573–6. doi: 10.1189/jlb.1008585
77. Berland R, Fernandez L, Kari E, Han JH, Lomakin I, Akira S, et al. Toll-like receptor 7-dependent loss of B cell tolerance in pathogenic autoantibody knockin mice. *Immunity* (2006) 25(3):429–40. doi: 10.1016/j.immuni.2006.07.014
78. Mobarrez F, Svenungsson E, Pisetsky DS. Microparticles as autoantigens in systemic lupus erythematosus. *Eur J Clin Invest* (2018) 48(12):e13010. doi: 10.1111/eci.13010
79. Christensen SR, Shupe J, Nickerson K, Kashgarian M, Flavell RA, Shlomchik MJ. Toll-like receptor 7 and TLR9 dictate autoantibody specificity and have opposing inflammatory and regulatory roles in a murine model of lupus. *Immunity* (2006) 25(3):417–28. doi: 10.1016/j.immuni.2006.07.013
80. Kang H-K, Liu M, Datta SK. Low-Dose Peptide Tolerance Therapy of Lupus Generates Plasmacytoid Dendritic Cells That Cause Expansion of Autoantigen-Specific Regulatory T Cells and Contraction of Inflammatory Th17 Cells. *J Immunol (Baltimore Md 1950)* (2007) 178(12):7849–58. doi: 10.4049/jimmunol.178.12.7849
81. Tilstra JS, John S, Gordon RA, Leibler C, Kashgarian M, Bastacky S, et al. B cell-intrinsic TLR9 expression is protective in murine lupus. *J Clin Invest* (2020) 130(6):3172–87. doi: 10.1172/jci132328
82. Singh RR, Ebling FM, Sercarz EE, Hahn BH. Immune tolerance to autoantibody-derived peptides delays development of autoimmunity in murine lupus. *J Clin Invest* (1995) 96:2990–6. doi: 10.1172/JCI118371
83. Sharabi A, Zinger H, Zborowsky M, Sthoeger ZM, Mozes E. A peptide based on the complementarity-determining region 1 of an autoantibody ameliorates lupus by up-regulating CD4+CD25+ cells and TGF-beta. *Proc Natl Acad Sci U S A* (2006) 103(23):8810–5. doi: 10.1073/pnas.0603201103
84. Aas-Hanssen K, Thompson KM, Bogen B, Munthe LA. Systemic Lupus Erythematosus: Molecular Mimicry between Anti-dsDNA CDR3 Idiotype, Microbial and Self Peptides-As Antigens for Th Cells. *Front Immunol* (2015) 6:382:382. doi: 10.3389/fimmu.2015.00382
85. Nyland JF, Stoll ML, Jiang F, Feng F, Gavalchin J. Mechanisms involved in the p62-73 idiopeptide-modulated delay of lupus nephritis in SNF(1) mice. *Lupus* (2012) 21(14):1552–64. doi: 10.1177/0961203312461964
86. Ghiggeri GM, D'Alessandro M, Bartolomeo D, Degl'Innocenti ML, Magnasco A, Lugani F, et al. An Update on Antibodies to Necleosome Components as Biomarkers of Sistemic Lupus Erythematosus and of Lupus Flares. *Int J Mol Sci* (2019) 20(22):5799. doi: 10.3390/ijms20225799
87. Ravirajan CT, Rowse L, MacGowan JR, Isenberg DA. An analysis of clinical disease activity and nephritis-associated serum autoantibody profiles in patients with systemic lupus erythematosus: a cross-sectional study. *Rheumatol (Oxford England)* (2001) 40(12):1405–12. doi: 10.1093/rheumatology/40.12.1405
88. Takahashi S, Fossati L, Iwamoto M, Merino R, Motta R, Kobayakawa T, et al. Imbalance towards Th1 predominance is associated with acceleration of lupus-like autoimmune syndrome in MRL mice. *J Clin Invest* (1996) 97(7):1597–604. doi: 10.1172/jci118584
89. Jackson SW, Jacobs HM, Arkatkar T, Dam EM, Scharping NE, Kolhatkar NS, et al. B cell IFN-γ receptor signaling promotes autoimmune germinal centers via cell-intrinsic induction of BCL-6. *J Exp Med* (2016) 213(5):733–50. doi: 10.1084/jem.20151724
90. Domeier PP, Chodisetti SB, Soni C, Schell SL, Elias MJ, Wong EB, et al. IFN-γ receptor and STAT1 signaling in B cells are central to spontaneous germinal center formation and autoimmunity. *J Exp Med* (2016) 213(5):715–32. doi: 10.1084/jem.20151722
91. 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 U S A* (2018) 115(38):9604–9. doi: 10.1073/pnas.1808594115
92. Roco JA, Mesin L, Binder SC, Nefzger C, Gonzalez-Figueroa P, Canete PF, et al. Class-Switch Recombination Occurs Infrequently in Germinal Centers. *Immunity* (2019) 51(2):337–50.e7. doi: 10.1016/j.immuni.2019.07.001
93. 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 (Baltimore Md 1950)* (2016) 197(5):1832–42. doi: 10.4049/jimmunol.1600143
94. Powell MD, Read KA, Sreekumar BK, Jones DM, Oestreich KJ. IL-12 signaling drives the differentiation and function of a T(H)1-derived T (FH1)-like cell population. *Sci Rep* (2019) 9(1):13991. doi: 10.1038/s41598-019-50614-1
95. Ma X, Nakayamada 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 Rheumatic Dis* (2018) 77(9):1354–61. doi: 10.1136/annrheumdis-2017-212652
96. 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
97. Facciotti F, Larghi P, Bosotti R, Vasco C, Gagliani N, Cordiglieri C, et al. Evidence for a pathogenic role of extrafollicular, IL-10-producing CCR6(+)B helper T cells in systemic lupus erythematosus. *Proc Natl Acad Sci U S A* (2020) 117(13):7305–16. doi: 10.1073/pnas.1917834117
98. Adams S, Zordan T, Sainis K, Datta SK. T cell receptor Vβ genes expressed by IgG anti-DNA autoantibody inducing T cells in lupus nephritis: Forbidden receptors and double negative T cells. *Eur J Immunol* (1990) 20:1435–43. doi: 10.1002/eji.1830200705
99. Crispin JC, Oukka M, Bayliss G, Cohen RA, Van Beek CA, Stillman IE, et al. Expanded double negative T cells in patients with systemic lupus erythematosus produce IL-17 and infiltrate the kidneys. *J Immunol* (2008) 181(12):8761–6. doi: 10.4049/jimmunol.181.12.8761
100. Holoshitz J, Koning F, Coligan JE, De Bruyn J, Strober S. Isolation of CD4-CD8- mycobacteria-reactive T lymphocyte clones from rheumatoid arthritis synovial fluid. *Nature* (1989) 339(6221):226–9. doi: 10.1038/339226a0
101. Kaliyaperumal A, Michaels MA, Datta SK. Naturally processed chromatin peptides reveal a major autoepitope that primes pathogenic T and B cells of lupus. *J Immunol* (2002) 168:2530–7. doi: 10.4049/jimmunol.168.5.2530
102. Lu L, Kaliyaperumal A, Boumpas DT, Datta SK. Major peptide autoepitopes for nucleosome-specific T cells of human lupus. *J Clin Invest* (1999) 104:345–55. doi: 10.1172/JCI6801
103. Stemmer C, Richalet-Secordel P, Van Bruggen MCJ, Kramers C, Berden JHM, Muller S. Dual reactivity of several monoclonal anti-nucleosome antibodies for double stranded DNA and a short segment of histone H3. *J Biol Chem* (1996) 271:21257–61. doi: 10.1074/jbc.271.35.21257
104. Panina-Bordignon P, Tan A, Termijtelen A, Corradin G, Lanzavecchia A. Universally immunogenic T cell epitopes: promiscous binding to human MHC class II and promiscous recognition by T cells. *Eur J Immunol* (1989) 19:2237–42. doi: 10.1002/eji.1830191209
105. Shi Y, Kaliyaperumal A, Lu L, Southwood S, Sette A, Michaels MA, et al. Promiscuous presentation and recognition of nucleosomal autoepitopes in lupus: Role of autoimmune T cell receptor alpha chain. *J Exp Med* (1998) 187:367–78. doi: 10.1084/jem.187.3.367

106. Wilson DB, Wilson DH, Schroder K, Pinilla C, Blondelle S, Houghten RA, et al. Specificity and degeneracy of T cells. *Mol Immunol* (2004) 40(14-15):1047–55. doi: 10.1016/j.molimm.2003.11.022
107. Michaels MA, Kang HK, Kaliyaperumal A, Satyaraj E, Shi Y, Datta SK. A defect in deletion of nucleosome-specific autoimmune T cells in lupus-prone thymus: role of thymic dendritic cells. *J Immunol* (2005) 175(9):5857–65. doi: 10.4049/jimmunol.175.9.5857
108. Kang H-K, Michaels MA, Berner BR, Datta SK. Very low-dose tolerance with nucleosomal peptides controls lupus and induces potent regulatory T cell subsets. *J Immunol* (2005) 174:3247–55. doi: 10.4049/jimmunol.174.6.3247
109. Kaliyaperumal A, Michaels MA, Datta SK. Antigen-specific therapy of murine lupus nephritis using nucleosomal peptides: Tolerance spreading impairs pathogenic function of autoimmune T and B cells. *J Immunol* (1999) 162:5775–83.
110. Kuttler C, Nussbaum AK, Dick TP, Rammensee HG, Schild H, Hadeler KP. An algorithm for the prediction of proteasomal cleavages (erratum in J. Mol. Biol. Aug. 4, 301:229). *J Mol Biol* (2000) 298:417–29. doi: 10.1006/jmbi.2000.3683
111. Rosalia RA, Quakkelaar ED, Redeker A, Khan S, Camps M, Drijfhout JW, et al. Dendritic cells process synthetic long peptides better than whole protein, improving antigen presentation and T-cell activation. *Eur J Immunol* (2013) 43(10):2554–65. doi: 10.1002/eji.201343324
112. Ghali JR, Holdsworth SR, Kitching AR. Targeting IL-17 and IL-23 in Immune Mediated Renal Disease. *Curr Med Chem* (2015) 22(38):4341–65. doi: 10.2174/0929867322666151030163022
113. Kitching AR, Holdsworth SR. The emergence of TH17 cells as effectors of renal injury. *J Am Soc Nephrol JASN* (2011) 22(2):235–8. doi: 10.1681/asn.2010050536
114. Stremska ME, Dai C, Venkatadri R, Wang H, Sabapathy V, Kumar G, et al. IL233, an IL-2-IL-33 hybrid cytokine induces prolonged remission of mouse lupus nephritis by targeting Treg cells as a single therapeutic agent. *J Autoimmun* (2019) 102:133–41. doi: 10.1016/j.jaut.2019.05.005
115. Rose A, von Spee-Mayer C, Kloke L, Wu K, Kühl A, Enghard P, et al. IL-2 Therapy Diminishes Renal Inflammation and the Activity of Kidney-Infiltrating CD4+ T Cells in Murine Lupus Nephritis. *Cells* (2019) 8 (10):1234. doi: 10.3390/cells8101234
116. Yan JJ, Lee JG, Jang JY, Koo TY, Ahn C, Yang J. IL-2/anti-IL-2 complexes ameliorate lupus nephritis by expansion of CD4(+)CD25(+)Foxp3(+) regulatory T cells. *Kidney Int* (2017) 91(3):603–15. doi: 10.1016/j.kint.2016.09.022
117. Chan OT, Madaio MP, Shlomchik MJ. The central and multiple roles of B cells in lupus pathogenesis. *Immunol Rev* (1999) 169:107–21. doi: 10.1111/j.1600-065x.1999.tb01310.x
118. Furie R, Rovin BH, Houssiau F, Malvar A, Teng YKO, Contreras G, et al. Two-Year, Randomized, Controlled Trial of Belimumab in Lupus Nephritis. *New Engl J Med* (2020) 383(12):1117–28. doi: 10.1056/NEJMoa2001180
119. Müller-Winkler J, Mitter R, Rappe JCF, Vanes L, Schweighoffer E, Mohammadi H, et al. Critical requirement for BCR, BAFF, and BAFFR in memory B cell survival. *J Exp Med* (2021) 218(2):e20191393. doi: 10.1084/jem.20191393
120. Datta SK, Kaliyaperumal A. Nucleosome-driven autoimmune response in lupus - Pathogenic T helper cell epitopes and costimulatory signals. In "B Lymphocytes and Autoimmunity" (Editors, N. Chiorazzi, R. G. Lahita, K. Pavelka, M. Ferrarini). *Ann N Y Acad Sci* (1997) 815:155–70. doi: 10.1111/j.1749-6632.1997.tb52057.x
121. Salinas VH, Stüve O. Systems Approaches to Unravel T Cell Function and Therapeutic Potential in Autoimmune Disease. *J Immunol (Baltimore Md 1950)* (2021) 206(4):669–75. doi: 10.4049/jimmunol.2000954
122. Wu HY, Ward FJ, Staines NA. Histone Peptide-induced nasal tolerance: suppression of murine lupus. *J Immunol* (2002) 169:1126–34. doi: 10.4049/jimmunol.169.2.1126
123. Wu HY, Monsonego A, Weiner HL. The mechanism of nasal tolerance in lupus prone mice is T-cell anergy induced by immature B cells that lack B7 expression. *J Autoimmun* (2006) 26(2):116–26. doi: 10.1016/j.jaut.2005.11.005
124. Mitchison NA. Induction of immunological paralysis in two zones of dosage. *Proc R Soc London B* (1964) 161:275–92. doi: 10.1098/rspb.1964.0093
125. Dieker J, Berden JH, Bakker M, Briand JP, Muller S, Voll R, et al. Autoantibodies against Modified Histone Peptides in SLE Patients Are Associated with Disease Activity and Lupus Nephritis. *PloS One* (2016) 11 (10):e0165373. doi: 10.1371/journal.pone.0165373
126. Daniel C, Weigmann B, Bronson R, von Boehmer H. Prevention of type 1 diabetes in mice by tolerogenic vaccination with a strong agonist insulin mimetope. *J Exp Med* (2011) 208(7):1501–10. doi: 10.1084/jem.20110574
127. Bergman ML, Lopes-Carvalho T, Martins AC, Grieco FA, Eizirik DL, Demengeot J. Tolerogenic insulin peptide therapy precipitates type 1 diabetes. *J Exp Med* (2017) 214(7):2153–6. doi: 10.1084/jem.20160471
128. Papillion A, Powell MD, Chisolm DA, Bachus H, Fuller MJ, Weinmann AS, et al. Inhibition of IL-2 responsiveness by IL-6 is required for the generation of GC-T(FH) cells. *Sci Immunol* (2019) 4(39):eaaw7636. doi: 10.1126/sciimmunol.aaw7636
129. Ballesteros-Tato A, Papillion A. Mechanisms of action of low-dose IL-2 restoration therapies in SLE. *Curr Opin Immunol* (2019) 61:39–45. doi: 10.1016/j.coi.2019.07.003
130. Balomenos D, Rumold R, Theofilopoulos AN. Interferon-gamma is required for lupus-like disease and lymphoaccumulation in MRL-lpr mice. *J Clin Invest* (1998) 101:364–71. doi: 10.1172/JCI750
131. Voll RE, Roth EA, Girkontaite I, Fehr H, Herrman M, Lorenz HM, et al. Histone-specific Th0 and Th1 clones derived from systemic lupus erythematosus patients induce double-stranded DNA antibody production. *Arthritis Rheumatol* (1997) 40:2162–71. doi: 10.1002/art.1780401210
132. Bruns A, Blass S, Hausdorf G, Burmester GR, Hiepe F. Nucleosomes are major T and B cell autoantigens in systemic lupus erythematosus. *Arthritis Rheumatol* (2000) 43:2307–15. doi: 10.1002/1529-0131(200010)43:10<2307::AID-ANR19>3.0.CO;2-J
133. Hoffman RW, Takeda Y, Sharp GC, Lee DR, Hill DL, Kaneoka H, et al. Human T cell clones reactive against U-snRNP autoantigens from connective tissue disease patients and healthy individuals. *J Immunol* (1993) 151:6460–9.
134. Cassotta A, Paparoditis P, Geiger R, Mettu RR, Landry SJ, Donati A, et al. Deciphering and predicting CD4+ T cell immunodominance of influenza virus hemagglutinin. *J Exp Med* (2020) 217(10):e20200206. doi: 10.1084/jem.20200206
135. Zhang L, Bertucci AM, Ramsey-Goldman R, Burt RK, Datta SK. Regulatory T cell (Treg) subsets return in patients with refractory lupus following stem cell transplantation, and TGF-β producing CD8+ Treg cells are associated with immunological remission of lupus. *J Immunol* (2009) 183(10):6346–58. doi: 10.4049/jimmunol.0901773
136. Geginat J, Vasco M, Gerosa M, Tas SW, Pagani M, Grassi F, et al. IL-10 producing regulatory and helper T-cells in systemic lupus erythematosus. *Semin Immunol* (2019) 44:101330. doi: 10.1016/j.smim.2019.101330
137. Singh RP, La Cava A, Hahn BH. pConsensus peptide induces tolerogenic CD8+ T cells in lupus-prone (NZB x NZW)F1 mice by differentially regulating Foxp3 and PD1 molecules. *J Immunol* (2008) 180(4):2069–80. doi: 10.4049/jimmunol.180.4.2069
138. Zheng SG, Wang JH, Koss MN, Quismorio FJr., Gray JD, Horwitz DA. CD4+ and CD8+ regulatory T cells generated ex vivo with IL-2 and TGF-beta suppress a stimulatory graft-versus-host disease with a lupus-like syndrome. *J Immunol* (2004) 172(3):1531–9. doi: 10.4049/jimmunol.172.3.1531
139. Beres AJ, Haribhai D, Chadwick AC, Gonyo PJ, Williams CB, Drobyski WR. CD8+ Foxp3+ regulatory T cells are induced during graft-versus-host disease and mitigate disease severity. *J Immunol (Baltimore Md 1950)* (2012) 189 (1):464–74. doi: 10.4049/jimmunol.1200886
140. Horwitz DA, Bickerton S, Koss M, Fahmy TM, La Cava A. Suppression of Murine Lupus by CD4+ and CD8+ Treg Cells Induced by T Cell-Targeted Nanoparticles Loaded With Interleukin-2 and Transforming Growth Factor β. *Arthritis Rheumatol (Hoboken NJ)* (2019) 71(4):632–40. doi: 10.1002/art.40773
141. Choi JY, Eskandari SK, Cai S, Sulkaj I, Assaker JP, Allos H, et al. Regulatory CD8 T cells that recognize Qa-1 expressed by CD4 T-helper cells inhibit rejection of heart allografts. *Proc Natl Acad Sci U S A* (2020) 117(11):6042–6. doi: 10.1073/pnas.1918950117
142. Saligrama N, Zhao F, Sikora MJ, Serratelli WS, Fernandes RA, Louis DM, et al. Opposing T cell responses in experimental autoimmune

encephalomyelitis. *Nature* (2019) 572(7770):481–7. doi: 10.1038/s41586-019-1467-x
143. Tang X, Kumar V. Advances in the Study of CD8+ Regulatory T Cells. *Crit Rev Immunol* (2019) 39(6):409–21. doi: 10.1615/CritRevImmunol.2020033260
144. 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 (5):2010–5. doi: 10.1073/pnas.1018974108
145. Mishra S, Liao W, Liu Y, Yang M, Ma C, Wu H, et al. TGF-β and Eomes control the homeostasis of CD8+ regulatory T cells. *J Exp Med* (2021) 218(1): e20200030. doi: 10.1084/jem.20200030
146. Minning S, Xiaofan Y, Anqi X, Bingjie G, Dinglei S, Mingshun Z, et al. Imbalance between CD8(+)CD28(+) and CD8(+)CD28(-) T-cell subsets and its clinical significance in patients with systemic lupus erythematosus. *Lupus* (2019) 28(10):1214–23. doi: 10.1177/0961203319867130
147. Zhang L, Bertucci AM, Ramsey-Goldman R, Harsha-Strong ER, Burt RK, Datta SK. Major pathogenic steps in human lupus can be effectively suppressed by nucleosomal histone peptide epitope-induced regulatory immunity. *Clin Immunol (Orlando Fla)* (2013) 149(3):365–78. doi: 10.1016/j.clim.2013.08.008
148. Zhong H, Liu Y, Xu Z, Liang P, Yang H, Zhang X, et al. TGF-β-Induced CD8 (+)CD103(+) Regulatory T Cells Show Potent Therapeutic Effect on Chronic Graft-versus-Host Disease Lupus by Suppressing B Cells. *Front Immunol* (2018) 9:35:35. doi: 10.3389/fimmu.2018.00035
149. Deng W, Xu M, Meng Q, Li Z, Qiu X, Yin S, et al. CD8+CD103+ iTregs inhibit the progression of lupus nephritis by attenuating glomerular endothelial cell injury. *Rheumatol (Oxford England)* (2019) 58(11):2039–50. doi: 10.1093/rheumatology/kez112
150. Keino H, Masli S, Sasaki S, Streilein JW, Stein-Streilein J. CD8+ T regulatory cells use a novel genetic program that includes CD103 to suppress Th1 immunity in eye-derived tolerance. *Invest Ophthalmol Visual Sci* (2006) 47 (4):1533–42. doi: 10.1167/iovs.04-1454
151. Uss E, Rowshani AT, Hooibrink B, Lardy NM, van Lier RAW, ten Berge IJM. CD103 Is a Marker for Alloantigen-Induced Regulatory CD8+ T Cells. *J Immunol* (2006) 177(5):2775–83. doi: 10.4049/jimmunol.177.5.2775
152. Tsai YG, Lee CY, Lin TY, Lin CY. $CD8^+$ Treg cells associated with decreasing disease activity after intravenous methylprednisolone pulse therapy in lupus nephritis with heavy proteinuria. *PloS One* (2014) 9(1):e81344. doi: 10.1371/journal.pone.0081344
153. Chaussabel D, Quinn C, Shen J, Patel P, Glaser C, Baldwin N, et al. A modular analysis framework for blood genomics studies: application to systemic lupus erythematosus. *Immunity* (2008) 29(1):150–64. doi: 10.1016/j.immuni.2008.05.012
154. Bave U, Magnusson M, Eloranta ML, Perers A, Alm GV, Ronnblom L. Fc gamma RIIa is expressed on natural IFN-alpha-producing cells (plasmacytoid dendritic cells) and is required for the IFN-alpha production induced by apoptotic cells combined with lupus IgG. *J Immunol (Baltimore Md 1950)* (2003) 171(6):3296–302. doi: 10.4049/jimmunol.171.6.3296
155. Baechler EC, Batliwalla FM, Karypis G, Gaffney PM, Ortmann WA, Espe KJ, et al. Interferon-inducible gene expression signature in peripheral blood cells of patients with severe lupus. *Proc Natl Acad Sci USA* (2003) 100:2610–5. doi: 10.1073/pnas.0337679100
156. Nikpour M, Dempsey AA, Urowitz MB, Gladman DD, Barnes DA. Association of a gene expression profile from whole blood with disease activity in systemic lupus erythaematosus. *Ann Rheumatic Dis* (2008) 67 (8):1069–75. doi: 10.1136/ard.2007.074765
157. Singh RR, Ebling FM, Albuquerque DA, Saxena V, Kumar V, Giannini EH, et al. Induction of autoantibody production is limited in nonautoimmune mice. *J Immunol* (2002) 169(1):587–94. doi: 10.4049/jimmunol.169.1.587
158. Kang HK, Chiang MY, Liu M, Ecklund D, Datta SK. The Histone Peptide H4 (71-94) Alone Is More Effective than a Cocktail of Peptide Epitopes in Controlling Lupus: Immunoregulatory Mechanisms. *J Clin Immunol* (2011) 31(3):379–94. doi: 10.1007/s10875-010-9504-4
159. Datta SK. Major peptide autoepitopes for nucleosome-centered T and B cell interaction in human and murine lupus. *Ann NY Acad Sci* (2003) 987:79–90. doi: 10.1111/j.1749-6632.2003.tb06035.x
160. Wardemann H, Yurasov S, Schaefer A, Young JW, Meffre E, Nussenzweig MC. Predominant autoantibody production by early human B cell precursors. *Sci (N Y NY)* (2003) 301(5638):1374–7. doi: 10.1126/science.1086907
161. Steinman L, Ho PP, Robinson WH, Utz PJ, Villoslada P. Antigen-specific tolerance to self-antigens in protein replacement therapy, gene therapy and autoimmunity. *Curr Opin Immunol* (2019) 61:46–53. doi: 10.1016/j.coi.2019.07.011
162. Serra P, Santamaria P. Antigen-specific therapeutic approaches for autoimmunity. *Nat Biotechnol* (2019) 37(3):238–51. doi: 10.1038/s41587-019-0015-4
163. Chataway J, Martin K, Barrell K, Sharrack B, Stolt P, Wraith DC. Effects of ATX-MS-1467 immunotherapy over 16 weeks in relapsing multiple sclerosis. *Neurology* (2018) 90(11):e955–62. doi: 10.1212/wnl.0000000000005118
164. Roep BO, Wheeler DCS, Peakman M. Antigen-based immune modulation therapy for type 1 diabetes: the era of precision medicine. *Lancet Diabetes Endocrinol* (2019) 7(1):65–74. doi: 10.1016/s2213-8587(18)30109-8
165. Walczak A, Siger M, Ciach A, Szczepanik M, Selmaj K. Transdermal application of myelin peptides in multiple sclerosis treatment. *JAMA Neurol* (2013) 70(9):1105–9. doi: 10.1001/jamaneurol.2013.3022
166. Lutterotti A, Yousef S, Sputtek A, Stürner KH, Stellmann JP, Breiden P, et al. Antigen-specific tolerance by autologous myelin peptide-coupled cells: a phase 1 trial in multiple sclerosis. *Sci Trans Med* (2013) 5(188):188ra75. doi: 10.1126/scitranslmed.3006168
167. Roep BO, Solvason N, Gottlieb PA, Abreu JRF, Harrison LC, Eisenbarth GS, et al. Plasmid-encoded proinsulin preserves C-peptide while specifically reducing proinsulin-specific $CD8^+$ T cells in type 1 diabetes. *Sci Trans Med* (2013) 5(191):191ra82. doi: 10.1126/scitranslmed.3006103
168. Suen J-L, Chuang Y-H, Tsai B-Y, Yau PM, Chiang B-L. Treatment of murine lupus using nucleosomal T cell epitopes identified by bone marrow-derived dendritic cells. *Arthritis Rheumatol* (2004) 50:3250–9. doi: 10.1002/art.20520
169. Linker-Israeli M, Bakke AC, Kitridou RC, Gendler S, Gillis S, Horwitz DA. Defective production of interleukin 1 and interleukin 2 in patients with systemic lupus erythematosus (SLE). *J Immunol (Baltimore Md 1950)* (1983) 130(6):2651–5.
170. Mizui M, Tsokos GC. Low-Dose IL-2 in the Treatment of Lupus. *Curr Rheumatol Rep* (2016) 18(11):68. doi: 10.1007/s11926-016-0617-5
171. Zhang L, Datta SK. Prospects for a peptide vaccine for human lupus. *Int J Clin Rheumatol* (2014) 9(3):229–32. doi: 10.2217/ijr.14.37
172. Hemmers S, Schizas M, Azizi E, Dikiy S, Zhong Y, Feng Y, et al. IL-2 production by self-reactive CD4 thymocytes scales regulatory T cell generation in the thymus. *J Exp Med* (2019) 216(11):2466–78. doi: 10.1084/jem.20190993
173. 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(1):121–36. doi: 10.1084/jem.20131142
174. Gutierrez-Ramos JC, Andreu JL, Revilla Y, Viñuela E, Martinez C. Recovery from autoimmunity of MRL/lpr mice after infection with an interleukin-2/vaccinia recombinant virus. *Nature* (1990) 346(6281):271–4. doi: 10.1038/346271a0
175. Humrich JY, Morbach H, Undeutsch R, Enghard P, Rosenberger S, Weigert O, et al. Homeostatic imbalance of regulatory and effector T cells due to IL-2 deprivation amplifies murine lupus. *Proc Natl Acad Sci U S A* (2010) 107 (1):204–9. doi: 10.1073/pnas.0903158107
176. He J, Zhang R, Shao M, Zhao X, Miao M, Chen J, et al. Efficacy and safety of low-dose IL-2 in the treatment of systemic lupus erythematosus: a randomised, double-blind, placebo-controlled trial. *Ann Rheumatic Dis* (2020) 79(1):141–9. doi: 10.1136/annrheumdis-2019-215396
177. Rosenzwajg M, Lorenzon R, Cacoub P, Pham HP, Pitoiset F, El Soufi K, et al. Immunological and clinical effects of low-dose interleukin-2 across 11 autoimmune diseases in a single, open clinical trial. *Ann Rheumatic Dis* (2019) 78(2):209–17. doi: 10.1136/annrheumdis-2018-214229
178. Cari L, De Rosa F, Nocentini G, Riccardi C. Context-Dependent Effect of Glucocorticoids on the Proliferation, Differentiation, and Apoptosis of Regulatory T Cells: A Review of the Empirical Evidence and Clinical Applications. *Int J Mol Sci* (2019) 20(5):1142. doi: 10.3390/ijms20051142
179. Kim D, Nguyen QT, Lee J, Lee SH, Janocha A, Kim S, et al. Anti-inflammatory Roles of Glucocorticoids Are Mediated by Foxp3(+)

Regulatory T Cells via a miR-342-Dependent Mechanism. *Immunity* (2020) 53(3):581–96. doi: 10.1016/j.immuni.2020.07.002
180. Pasare C, Medzhitov R. Toll pathway-dependent blockade of $CD4^+CD25^+$ T cell-mediated suppression by dendritic cells. *Sci (N Y NY)* (2003) 299:1033–6. doi: 10.1126/science.1078231
181. Fadok VA, Bratton DL, Konowal A, Freed PW, Westcott JY, Henson PM. Macrophages that have ingested apoptotic cells in vitro inhibit proinflammatory cytokine production through autocrine/paracrine mechanisms involving TGF-beta, PGE2, and PAF. *J Clin Invest* (1998) 101:890–8. doi: 10.1172/JCI1112
182. Travis MA, Reizis B, Melton AC, Masteller E, Tang Q, Proctor JM, et al. Loss of integrin alpha(v)beta8 on dendritic cells causes autoimmunity and colitis in mice. *Nature* (2007) 449(7160):361–5. doi: 10.1038/nature06110
183. Vahl JC, Drees C, Heger K, Heink S, Fischer JC, Nedjic J, et al. Continuous T cell receptor signals maintain a functional regulatory T cell pool. *Immunity* (2014) 41(5):722–36. doi: 10.1016/j.immuni.2014.10.012
184. Rubtsov YP, Niec RE, Josefowicz S, Li L, Darce J, Mathis D, et al. Stability of the regulatory T cell lineage in vivo. *Sci (N Y NY)* (2010) 329(5999):1667–71. doi: 10.1126/science.1191996
185. Kishimoto TK. Development of ImmTOR Tolerogenic Nanoparticles for the Mitigation of Anti-drug Antibodies. *Front Immunol* (2020) 11:969. doi: 10.3389/fimmu.2020.00969
186. Do MH, Wang X, Zhang X, Chou C, Nixon BG, Capistrano KJ, et al. Nutrient mTORC1 signaling underpins regulatory T cell control of immune tolerance. *J Exp Med* (2020) 217(1):e20190848. doi: 10.1084/jem.20190848
187. Valle A, Jofra T, Stabilini A, Atkinson M, Roncarolo MG, Battaglia M. Rapamycin prevents and breaks the anti-CD3-induced tolerance in NOD mice. *Diabetes* (2009) 58(4):875–81. doi: 10.2337/db08-1432
188. Lai ZW, Kelly R, Winans T, Marchena I, Shadakshari A, Yu J, et al. Sirolimus in patients with clinically active systemic lupus erythematosus resistant to, or intolerant of, conventional medications: a single-arm, open-label, phase 1/2 trial. *Lancet (London England)* (2018) 391(10126):1186–96. doi: 10.1016/s0140-6736(18)30485-9
189. Zhang YN, Poon W, Tavares AJ, McGilvray ID, Chan WCW. Nanoparticle-liver interactions: Cellular uptake and hepatobiliary elimination. *J Controlled Release Off J Controlled Release Soc* (2016) 240:332–48. doi: 10.1016/j.jconrel.2016.01.020
190. Miao X, Leng X, Zhang Q. The Current State of Nanoparticle-Induced Macrophage Polarization and Reprogramming Research. *Int J Mol Sci.* (2017) 18(2):336 doi: 10.3390/ijms18020336
191. Ganugula R, Arora M, Zou D, Agarwal SK, Mohan C, Kumar M. A highly potent lymphatic system-targeting nanoparticle cyclosporine prevents glomerulonephritis in mouse model of lupus. *Sci Adv* (2020) 6(24): eabb3900. doi: 10.1126/sciadv.abb3900
192. Akamatsu M, Mikami N, Ohkura N, Kawakami R, Kitagawa Y, Sugimoto A, et al. Conversion of antigen-specific effector/memory T cells into Foxp3-expressing T(reg) cells by inhibition of CDK8/19. *Sci Immunol* (2019) 4(40): eaaw2707. doi: 10.1126/sciimmunol.aaw2707
193. Mikami N, Kawakami R, Sakaguchi S. New Treg cell-based therapies of autoimmune diseases: towards antigen-specific immune suppression. *Curr Opin Immunol* (2020) 67:36–41. doi: 10.1016/j.coi.2020.07.004
194. Pompura SL, Wagner A, Kitz A, Laperche J, Yosef N, Dominguez-Villar M, et al. Oleic acid restores suppressive defects in tissue-resident FOXP3 regulatory T cells from patients with multiple sclerosis. *J Clin Invest* (2020) 131(2):e138519. doi: 10.1172/jci138519

Conflict of Interest: 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.

frontiers in Immunology

REVIEW
published: 27 April 2021
doi: 10.3389/fimmu.2021.667342

The Potential of Harnessing IL-2-Mediated Immunosuppression to Prevent Pathogenic B Cell Responses

Amber Papillion and André Ballesteros-Tato *

Division of Clinical Immunology and Rheumatology, Department of Medicine, University of Alabama at Birmingham, Birmingham, AL, United States

Immunosuppressive drugs can partially control Antibody (Ab)-dependent pathology. However, these therapeutic regimens must be maintained for the patient's lifetime, which is often associated with severe side effects. As research advances, our understanding of the cellular and molecular mechanisms underlying the development and maintenance of auto-reactive B cell responses has significantly advanced. As a result, novel immunotherapies aimed to restore immune tolerance and prevent disease progression in autoimmune patients are underway. In this regard, encouraging results from clinical and preclinical studies demonstrate that subcutaneous administration of low-doses of recombinant Interleukin-2 (r-IL2) has potent immunosuppressive effects in patients with autoimmune pathologies. Although the exact mechanism by which IL-2 induces immunosuppression remains unclear, the clinical benefits of the current IL-2-based immunotherapies are attributed to its effect on bolstering T regulatory (Treg) cells, which are known to suppress overactive immune responses. In addition to Tregs, however, rIL-2 also directly prevent the T follicular helper cells (Tfh), T helper 17 cells (Th17), and Double Negative (DN) T cell responses, which play critical roles in the development of autoimmune disorders and have the ability to help pathogenic B cells. Here we discuss the broader effects of rIL-2 immunotherapy and the potential of combining rIL-2 with other cytokine-based therapies to more efficiently target Tfh cells, Th17, and DN T cells and subsequently inhibit auto-antibody (ab) production in autoimmune patients.

Keywords: IL-2 (interleukin-2), Tfh and immunity, autoimmune disease, Th17 & Tregs cells, auto-antibodies

OPEN ACCESS

Edited by:
David A. Horwitz, University of Southern California, United States

Reviewed by:
Bergithe Eikeland Oftedal, University of Bergen, Norway
Matthew Cook, Australian National University, Australia

***Correspondence:**
André Ballesteros-Tato
andreballesterostato@uabmc.edu

Specialty section:
This article was submitted to Immunological Tolerance and Regulation, a section of the journal Frontiers in Immunology

Received: *12 February 2021*
Accepted: *06 April 2021*
Published: *27 April 2021*

Citation:
Papillion A and Ballesteros-Tato A (2021) The Potential of Harnessing IL-2-Mediated Immunosuppression to Prevent Pathogenic B Cell Responses. Front. Immunol. 12:667342. doi: 10.3389/fimmu.2021.667342

INTRODUCTION

Self-reactive auto-antibodies (auto-Abs) against nuclear and cytoplasmic antigens play critical roles in autoimmune disease development and severity (1, 2). Auto-Abs contribute to disease pathogenesis by direct and indirect mechanisms. On the one hand, immune complexes (IC) formed by Auto-Abs and self-antigens activate antigens presenting cells and innate cells through the activation of Fc receptors (FCRs), thereby initiating a feedback loop of immune activation that ultimately leads to unwarranted inflammation and tolerance breakdown (3–5). Auto-Abs also engage the complement system, which

mediates tissue damage and further contributes to triggering systemic inflammation. Deposition of IC in the blood vessels, kidney, joints, and lungs amplifies the local inflammatory response and enhance tissue damage. In agreement with their pathogenic roles, the serum levels of auto-Abs strongly correlate with disease activity and severity in multiple forms of autoimmune disease, including systemic lupus erythematosus (SLE), type 1 diabetes (T1D), or rheumatoid arthritis (RA), among others. Furthermore, Auto-Abs can be detected years before the onset of clinical manifestations (6), thus suggesting that loss of B cell tolerance and production of Auto-Abs is a critical step that precedes the development of the autoimmune disease. While Ab-dependent pathology can be partially controlled by immunosuppression, there is currently no cure for systemic autoimmune disorders.

Auto-Abs are produced by autoreactive-plasma cells (PCs), a subset of terminally differentiated B cells that secrete large amounts of Abs (7). PCs can be originated in the germinal centers (GCs), the site of B cell maturation, where B cells undergo rapid rounds of proliferation, somatic hypermutation, and affinity maturation leading to the generation of high-affinity antibodies (8–10). Autoreactive PCs can also be generated outside the GCs *via* the extrafollicular pathway (11–13). Recent studies demonstrate a critical role for the extrafollicular PCs in the development of pathogenic Ab responses (11, 13, 14).

In the last decade, B cell depleting therapies were designed based on the rationale that depletion of self-reactive B cells would reduce the production of auto-Ab and subsequent Auto-Ab-mediated immunopathology (15, 16). However, the clinical efficacy of these therapies is lower than initially anticipated (17–19). The inability of B cell depleting agents to eliminate self-reactive PCs efficiently has been suggested as a plausible explanation for the relatively low effectiveness of these approaches (17). The life-threatening side effects of sustained immunosuppression and the failure of new therapies, such as B cell depletion, vindicate looking for new therapeutic alternatives to treat Ab-mediated pathologies. In this manuscript, we review the potential of low-dose IL-2-based immunotherapies to target T cell populations with B cell helper activity, mainly T follicular helper cells (Tfh), T helper 17 (Th17) cells, and Double-negative (DN) CD3+CD4-CD8- T cells (**Figure 1**). While IL-2 also induces immunosuppression by Treg-dependent mechanisms, more extensive reviews on this topic are available elsewhere. Hence, the role of IL-2 in promoting Treg-mediated immunosuppression will be only briefly discussed in this review.

PATHOGENIC B CELL HELPER T CELL SUBSETS

Tfh Cells

T follicular helper (Tfh) cells are a subset of $CD4^+$ T cells that provide co-stimulatory signals and cytokines that are required for the development and maintenance of GCs (20–22) and extrafollicular PC differentiation (23, 24) **(Figure 2)**. In the absence of pathogen-specific Tfh cells, GCs do not develop, and

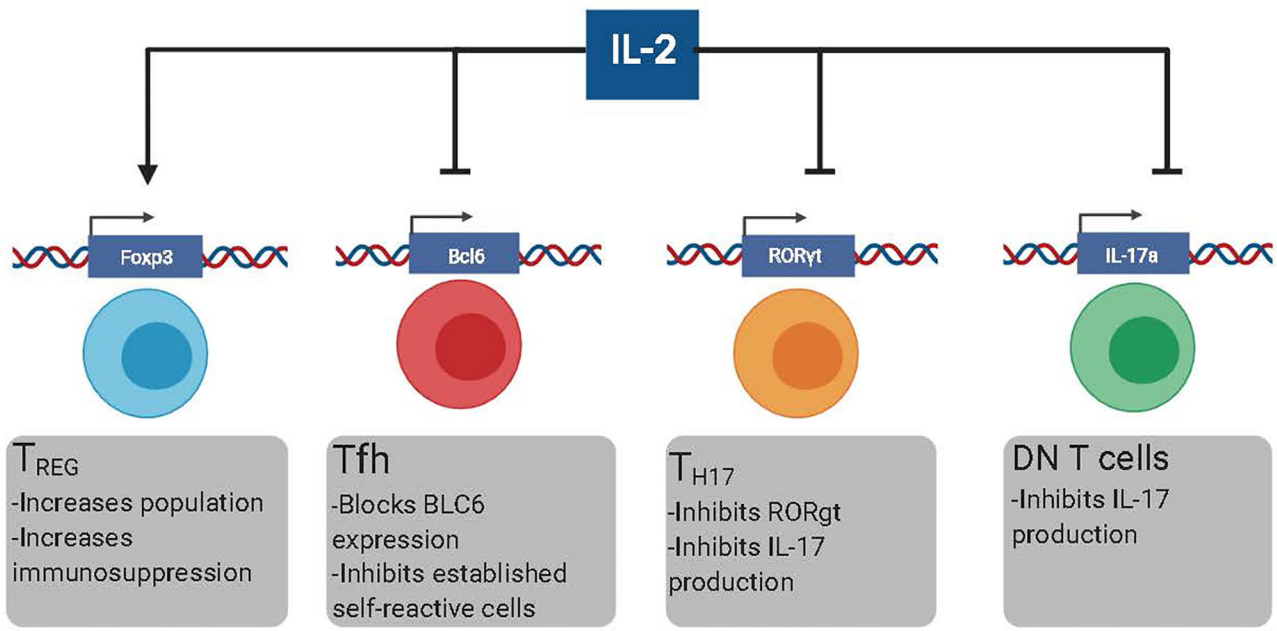


FIGURE 1 | The different effects of low dose rIL-2 therapy in autoimmunity. Low dose rIL-2 stabilizes FoxP3 program in Treg cells which increases both the size of the population and enhances immunosuppression. Low dose rIL-2 therapy can both inhibit the generation of new self-reactive Tfh cells and decrease already present self-reactive Tfh cells by blocking Bcl6. IL-6 blockade will make Tfh cells more suspectable to IL-2 signaling. Low dose rIL-2 can inhibit Th17 cells by diminishing expression of RORgt and inhibiting *IL17a* expression. Low dose rIL2 can also inhibit IL-17 production by DN T cells by directly inhibiting *IL17a*.

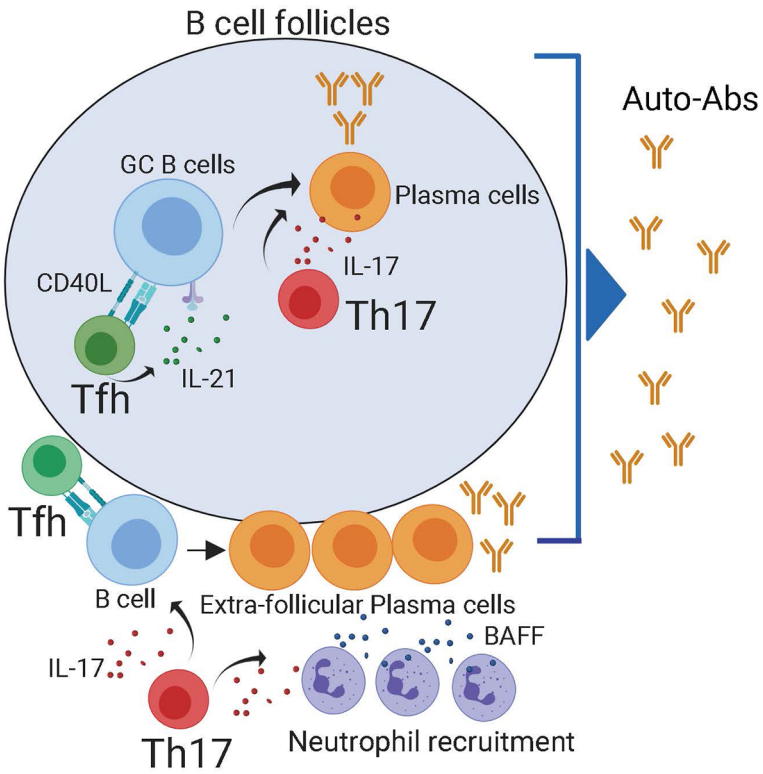


FIGURE 2 | Both follicular and extra-follicular pathways contribute to auto-antibody production. Within the B cell follicles, Tfh cells maintain auto-reactive germinal center reactions which generate self-reactive plasma cells. Tfh cells can also provide help in to extra-follicular PCs in the B cell border. Reports have demonstrated that IL-21 produced by Th17 cells can also contribute to the production of self-reactive plasma cells in the follicle. Besides, in the extra-follicular space, IL-17 produced by Th17 recruits BAFF producing neutrophils. The combination of IL-17, BAFF, and Tfh cells promotes the generation of self-reactive plasma cells.

pathogen-specific PC responses are impaired. Phenotypically, Tfh cells are characterized by the expression of CXCR5, PD1, ICOS, and Bcl6, among other markers (25). CXCR5 is a chemokine receptor that allows Tfh cells to localize in the proximity of the B/T cell border in response to CXCL13. The inhibitory receptor PD-1 and the co-stimulatory receptor ICOS ultimately direct Tfh cells into the B cell follicles (26–28), where they provide CD40L (22, 29) and IL-21 (30–32) to the responding B cells. Bcl6 is a transcription factor that promotes the expression of genes required for Tfh cell development and function while preventing the up-regulation of transcription factors implicated in T effector (Teff) cell differentiation (33–35). Bcl6 is critical for the differentiation of Tfh cells (33–35). Thus, it is considered the master regulator of Tfh differentiation. While Bcl6 promotes Tfh formation, the transcription factor Blimp-1 represses it (33–35). Importantly, Blimp-1 and Bcl6 are mutually antagonistic transcription factors that directly repress one another in $CD4^+$ T cells. Thus, the balance between the relative expression of Bcl6 and Blimp-1, rather than the expression of Bcl6 alone, fine-tunes the commitment into the Tfh cell pathway (20).

Under homeostatic conditions, Tfh cells help germinal center B cells to facilitate somatic hypermutation and class switch to generate long-term Ab protection to pathogens (25). However, when there is a break in intolerance, self-reactive Tfh cells are not depleted from the repertoire and provide co-stimulatory signals and cytokines to self-reactive B cells, leading to pathogenic auto-Ab responses (36, 37). In agreement, the presence of Tfh cells correlates with elevated levels of Auto-Ab and disease activity in preclinical animal models and autoimmune patients (37–41). When Tfh cells are depleted or decreased, autoimmune disease pathogenesis and auto-Ab responses are reduced (40, 42, 43). Based on these findings, Tfh cells are considered a potential target for autoimmune disorders (44). However, to date, there are no therapeutic agents approved to selectively deplete Tfh cells *in vivo*.

Th17 Cells

Th17 cells are a specialized subset of $CD4^+$ T cells that play an essential role in aiding host defense by recruiting neutrophils and macrophages. Th17 cells differentiate in response to TGFβ and IL-6, and their development is driven by the transcription factor RORγt (45). Excessive Th17 cell responses are implicated in the pathogenesis of multiple forms of autoimmune diseases. As such, the expansion of self-reactive Th17 cells correlates with disease activity in common autoimmune diseases, including RA, psoriasis, asthma, and lupus (46–48). Due to its characteristic pro-inflammatory properties, it is generally believed that Th17 cells contribute to autoimmune disease pathogenesis by inducing

tissue inflammation. However, recent studies demonstrate that Th17 cells can also help the development of auto-reactive GC B cells (48–51) and extrafollicular PCs (52). In agreement with this idea, IL-17 deficiency prevents auto-Ab production and disease progression in lupus-prone mice (53–55). In addition, the self-reactive GC responses were reduced by IL-17 deficiency in autoimmune Roquin$^{san/san}$ mice, thereby suggesting a cause-effect relationship between IL-17 and pathogenic GC B cell responses. Collectively, these studies suggest an important role for IL-17 producing cells in promoting pathogenic-B cell responses in the context of autoimmune disorders.

The capacity of Th17 cells to help B cell responses is not entirely surprising since, like Tfh cells, Th17 characteristically produce large amounts of IL-21 (56), a cytokine that promotes GC and PC differentiation. Furthermore, IL-17 synergizes with the B cell-activating factor belonging to the TNF family, BAFF, to protect responding cells from BCR-induced apoptosis (57), demonstrating an intrinsic effect of IL-17 in promoting B cell survival. Besides, IL-17 promotes the recruitment of neutrophils (58, 59), which secrete both BAFF and APRIL and can facilitate the survival and devolvement of extrafollicular PCs (60, 61). Collectively, these studies suggest that IL-17-producing cells can, directly and indirectly, help auto-reactive B cells responses, thereby contributing to Ab-mediated pathology in autoimmune patients **(Figure 2)**. However, whether IL-17-producing cells promote rather than merely correlate with self-reactive B cell responses in autoimmune patients has not yet been formally demonstrated.

Hybrid IL-17$^+$Tfh Cells?

An additional important question remaining is how the putative *"IL-17$^+$ helper"* cells gain access to the B cell follicles to provide B cell help. One exciting possibility is that pathogenic *"IL-17$^+$ helper"* cells are indeed Tfh cells that secrete IL-17. In agreement with this possibility, studies suggest the presence of hybrid IL-17-producing cells with "Tfh-like" characteristics in autoimmune prone BXD2 mice (48) and human tonsils (45). Furthermore, the culture of human CD4$^+$ T cells with a combination of TGFβ and IL-23, which is frequently used for the *in vitro* differentiation of Th17 cells (45), triggers the acquisition of a Tfh-like transcriptional signature characterized by the up-regulation of Bcl6, c-Maf, and CXCR5, and the down-regulation of Blimp-1, thereby resulting in the acquisition of a hybrid Bcl6$^+$RORγt$^+$ Tfh/Th17 signature (62). Whether hybrid Tfh/Th17 cells are Tfh cells that secondary acquire the capacity of secrete IL-17 or represent a separate lineage of Tfh cells is still unclear. Further investigations are needed in order to clarify the potential relationship between these two lineages.

Interestingly, the 'pro-Tfh" effect of TGF-β is restricted to humans, as TGF-β does not significantly affect Tfh cell differentiation in mice (62, 63). Nevertheless, the commonality between the Tfh and Th17 differentiation requirements extends beyond TGF-β. For example, ICOS, which is required for the survival and the migration of Tfh cells into the B cell follicles (26, 28, 64), is also critical for the differentiation and maintenance of Th17 cells (65, 66). Besides, similar to Tfh cells, the IL-6/STAT3 pathway is also a key positive regulator of Th17 differentiation (67). Thus, critical signaling pathways implicated in Tfh cell differentiation also critically regulate the Th17 program. Therefore, it is reasonable to speculate that the same inflammatory conditions that promote Tfh differentiation in autoimmune patients also favor the development of Th17 cells and/or the generation of hybrid IL-17-producing Tfh cells.

The concept of a highly pathogenic hybrid Tfh/IL-17 population with superior helper activity is, however, at odds with early studies suggesting that Bcl6 functions as a direct transcriptional repressor that prevents the acquisition of Teff programs, including the Th17 program (33–35). Indeed, Bcl6^{hi}CXCR5^{hi}PD-1^{hi} Tfh cells present in the B cell follicles (we will refer to these cells as GC-Tfh cells) do not normally produce IL-17, which is consistent with studies showing that high-expression of Bcl6 in GC-Tfh cells directly represses RORγt and Th17 differentiation (33, 34). Nevertheless, the role of Bcl6 in controlling alternative differentiation programs in Tfh cells is puzzling. As such, while studies suggest that Bcl6 binds to the *Rorc* promoter and inhibits its expression (33), other studies show no evidence of Bcl6 binding (34, 68). Furthermore, while Bcl6-expressing cells do not normally express Teff cytokines, some studies indicate that Tfh cells can produce effector cytokines in the context of high inflammatory conditions, such as viral infections (69–72) or autoimmune diseases (48). In addition, extrafollicular-Tfh cells (which express medium levels of Bcl6) have a more heterogeneous transcriptional signature than Bcl6^{hi} GC-Tfh cells (which express high levels of Bcl6) (73–75). These results suggest that the ability of Bcl6 to inhibit the initiation of secondary Teff differentiation programs in developing Tfh cells is dose-dependent and can be partially overcome in highly reactive environments, such as in autoimmune diseases, thereby leading to the acquisition of hybrid Tfh/Teff phenotypes, such as IL-17$^+$ Tfh cells, with enhanced pathogenic functions.

Double-Negative T Cells

Double-negative (DN) CD3$^+$CD4$^-$CD8$^-$ T cells are a rare population of TCR-αβ$^+$ T cells that lack CD4 and CD8 expression and express high levels of B220 (76, 77). While DN T cells are relatively scarce in healthy individuals, they abnormally expand in lupus patients and children with autoimmune diseases, such as mixed connective tissue disease or juvenile idiopathic arthritis (78). Aberrant accumulation of DN T cells is also a clinical hallmark of the Autoimmune Lymphoproliferative Syndrome (ALPS, also known as Canale-Smith syndrome), a genetic disorder caused by defective FAS-mediated apoptosis that is characterized by the development of autoimmune disease, splenomegaly, lymphadenopathy, and an increased risk of secondary lymphomas during childhood (79, 80). In aged MRL/*lpr* mice, DN T cells represent nearly 70% of the total cells in the enlarged lymph nodes, accounting for the characteristic lymphadenopathy observed in these mice.

The exact origin of DN T cells remains controversial (76, 77). Early studies suggest they derive from activated CD4 T cells that fail to undergo apoptosis (81). However, a more detailed examination of the DN T cell origin *in vivo* indicates that DN T cells derive from CD8$^+$ T cells that down-regulate their co-receptor after continuous stimulation by self-antigens derived

from apoptotic cells (82, 83). Thus, it is generally believed that DN T cells derive from $CD8^+$ T cells.

Expansion of DN T cells correlates with disease activity in lupus-prone mice (83, 84) and systemic SLE patients (85), leading to the idea that these cells play an important pathogenic role in autoimmune disease development. Despite the evidence supporting a pathogenic role for DN T cells, their exact function remains largely elusive (76, 77). Interestingly, DN T cells express high levels of CXCR5, localize in the B cells follicles (86), and stimulate Ab production *in vitro* (85, 87). Moreover, similar to Tfh cells, the presence of DN T cells correlates with disease activity and autoantibody production in SLE patients and MRL.lpr mice (76, 77, 83–85, 88). Moreover, new studies show that DN T cells produce large amounts of IL-17 (54, 83, 88). Given the putative role of IL-17 in helping B cell responses (48–52, 56, 89), it is tempting to speculate that $CXCR5^+$DN T cells contribute to autoimmune pathology by promoting auto-reactive B cell responses in an IL-17-dependent manner. Corresponding with this idea, a recent study demonstrated that DN T cells are sufficient to promote autoantibody production and renal immune complex deposition after adoptive transfer into B6 *Rag1*$^{-/-}$ mice that also received B cells from 12-month-old B6.*lpr* mice (83). These findings provide evidence that DN T cells can contribute to pathogenic Ab responses *in vivo*. Further investigations will be required to compare the capacity of DN T cells, Tfh cells, and bona fide Th17 to help self-reactive B cell responses and determine how each of these subsets relatively contribute to sustaining pathogenic-Ab responses.

IL-2 AND IMMUNOSUPPRESSION

IL-2 Signaling

IL-2 is a member of the common γ-chain family of cytokines that was initially characterized and as a growth factor for T and NK T cells (90–92). IL-2 signaling is transmitted through the IL-2 receptor (IL-2R), which can exist in two conformations (93). The high-affinity receptor is a heterotrimeric receptor that consists of the α chain (CD25), the β chain (CD122), and the common γ chain (CD132) (94, 95). The high-affinity receptor is constitutively expressed by FoxP3-expressing $CD4^+$ regulatory T-cells (Tregs), which require IL-2 signaling for their differentiation and function (96–99). In contrast, NK T cells, naïve and memory T cells express the intermediate-affinity IL-2R, a heterodimer composed of the β and γ chain. Following TCR activation, however, they transiently up-regulate CD25 and temporarily express the high-affinity IL-2R. The differential expression of CD25 by regulatory T cells and conventional T cells has important therapeutic consequences. When administered at high doses, IL-2 can help conventional T cells and NK T cells, hence favoring effector responses. In contrast, because Tregs express high levels of CD25 and better compete for IL-2 than other cells, low IL-2 regimes preferentially target IL-2 to Tregs, thus promoting immunosuppression (100).

The binding of IL-2 to the IL-2R triggers the phosphorylation of the Janus-Activated Kinase 1 (JAK1) and 3 (JAK3), leading to the activation of the transcription factor STAT5 (101). In addition, phosphorylation of the adaptor Shc in response to IL-2 activates the Ras-Raf MAP Kinase and PI-3K pathways. The combined effects of STAT5, Ras-Raf MAP Kinase, and PI-3K signaling pathways results in the regulation of the transcription of a broad range of IL-2-target genes, including the forkhead box P3 (FOXP3) (102–104), eomesodermin (Eomes) (105), the B Lymphocyte Induced Maturation Protein 1 (BLIMP1) (105), the T-box transcription factor TBX21 (T-bet) (106, 107), Retinoic acid-related Orphan Receptor (ROR)γ (108–110) and B cell lymphoma (Bcl6) (107, 111–114). Due to its pleiotropic transcriptional effects, IL-2 has been implicated in regulating multiple, and often contradictory, critical immunoregulatory pathways. For example, IL-2–STAT5 signaling positively regulates IL-4R and GATA-3 expression and subsequent Th2 differentiation (106). On the other hand, IL-2 induces IL-12Rβ2, Blimp-1, and IFN-γ up-regulation, which are required for Th1 cell polarization (106).

Importantly, while IL-2 signaling can help effector responses, the development of a lethal multiorgan autoimmune syndrome in the IL-2 and IL-2R deficient mice revealed that the critical non-redundant function of IL-2 is to promote immunosuppression (115–118). Rather than immunodeficiency, diminished IL-2 production is associated with autoimmune disease development in mice and humans (119–123), highlighting the critical role of this cytokine in maintaining immunological tolerance. Given that Treg cells fail to normally develop in the absence of IL-2 signaling (98, 124–126) and that they are essential for maintaining immune tolerance (127–130), it is generally accepted that the principal mechanism by which IL-2 contributes to preserving immune tolerance is by supporting the development and function of Tregs. Supporting this view, early transfer of Tregs into neonatal CD122 deficient mice prevents autoimmune pathology (96).

Low-Dose IL-2 Therapy

Work done over the last fifteen years demonstrate the potential of leveraging the immunosuppressive properties of IL-2 to treat autoimmune disorders. Early studies show that exogenous IL-2 supplementation prevents disease progression and contributes to inducing immunosuppression in mice with established autoimmune diseases, including Type I diabetes, EA, experimental myasthenia, and lupus (131–138). More recently, a novel immunotherapy based on subcutaneous administration of low-dose recombinant human IL-2 (r-IL2, (Aldesleukin/Proleukin) has shown potent immunosuppressive effects in patients with autoimmune pathologies (139), including Type I diabetes (140), hepatitis C-associated vasculitis (141), SLE (142–145), and chronic graft-versus-host disease (146–148). The recent TRANSREG clinical trial further demonstrated that the same dose of rIL-2 selectively expands Tregs and clinical benefits across eleven selected autoimmune diseases (149). Collectively these studies demonstrate that low-dose rIL-2 regimes have therapeutic effects across a broad range of heterogonous autoimmune disorders.

Current low-dose rIL-2 treatment schemes consist of 3-4 cycles of 7-10 million IU of rIL-2 per cycle administered over 1-2 weeks separated by resting periods of 9-16 days. Importantly, low-dose rIL-2 can be safely administered to humans. Thus numerous clinical trials to further explore the potential benefits of low-dose IL-2 in SLE are now underway.

Based on the critical functional relationship between IL-2 and Treg-mediated immunosuppression, the current paradigm suggests that low-dose rIL-2 regimes contribute to restoring immune homeostasis in autoimmune patients by a Treg-dependent mechanism (139, 150). In agreement with this view, low-dose rIL-2 supplementation induces Treg cell expansion *in vivo* (139). Hence considerable effort has been invested in developing new therapeutic approaches to selectively target IL-2 to Tregs.

Intriguingly, though the frequency of Tregs increases after low-dose rIL-2 administration, the changes in Treg cell numbers are transient and drop to placebo control levels quickly after the last rIL-2 cycle (142, 143). Nevertheless, despite a nearly normal frequency of Tregs, the improved clinical outcomes persist for weeks after the last cycle of rIL-2 (142, 143). Thus, while it is clear that Treg-mediated immunosuppression is critical for achieving the clinical benefits observed after rIL-2 treatment, additional underlying mechanisms might synergize with Treg-mediated effects to provide long-lasting immunosuppression after low-dose rIL-2 immunotherapy. In this regard, recent studies demonstrate that prolonged IL-2 signaling prevents the expression of (RORγt) (110) and Bcl6 (107, 111–114), thereby repressing Th17 and Tfh cell development, respectively. Correspondingly, low-dose rIL-2 treatment significantly reduced the frequency of Tfh and Th17 in humans and preclinical animal models (71, 114, 138, 143, 151, 152). Similarly, $CD3^+CD4^-CD8^-$ DN T cells are depleted after rIL-2 administration (138). Based on the inhibitory role of IL-2 in Tfh, Th17, and DN T cells and their putative roles in promoting self-reactive B cell responses, targeting IL-2 to these T cell populations could represent a good therapeutic strategy to prevented Ab-mediated pathology in autoimmune patients without inducing profound immunosuppression.

TARGETING B CELL HELPERS WITH IL-2

IL-2 and Tfh Cells

Studies by us and others demonstrate that IL-2 signaling inhibits Tfh cell differentiation (107, 111, 113, 114, 152). Mechanistically, IL-2 indirectly inhibits Tfh cells by inducing BLIMP, which in turn represses Bcl6 expression and Tfh cell differentiation (111, 113). Besides, STAT5 in response to IL-2 binds to the Bcl6 promoter and directly prevents Bcl6 transcription (107, 112), thereby inhibiting the initiation of the Tfh cell program. In support of these findings, the lack of IL-2/STAT5 signaling during T cell differentiation skews the $CD4^+$ T cell response towards the Tfh cell differentiation pathway (111, 113, 114). Data from the Weinmann's laboratory also suggest that, in addition to directly repressing Bcl6 expression, IL-2 signaling favors the formation of T-bet/Bcl6 complexes that block Bcl6 activity (107).

Corresponding with the inhibitory role of IL-2 in Tfh cell development, Tfh cell differentiation can be fine-tuned *in vivo* by altering the environmental levels of IL-2. As such, limiting IL-2 signaling *in vivo* results in enhanced Tfh cell responses (111, 113, 114, 151–153). Contrariwise, treatment with rIL-2 prevents Tfh cell differentiation and ensuing GC responses in mice infected with influenza virus (71, 114, 151). Importantly, in these studies, the ability of IL-2 to suppress Tfh cell responses is independent of the presence of Tregs (114, 152). Hence, these studies demonstrate that lL-2 intrinsically inhibits Tfh cell development by repressing Bcl6 expression and activity in a Treg-independent manner.

Notably, there are significant differences between human and mouse Tfh cell developmental requirements (154, 155). However, recent studies demonstrate that IL-2 is also a potent inhibitor of human Tfh cell responses. Corresponding with this, while IL-2 blockade increases human Tfh cell differentiation *in vitro* (156), treatment with low-dose rIL-2 reduces the frequency of Tfh cells in SLE patients (143). These results provide evidence to support the potential of IL-2-based therapies to deplete Tfh cells *in vivo*. Furthermore, these studies offer a new interpretation for how impaired IL-2 production by T cells (120–123) and single nucleotide polymorphisms in the IL-2 and IL-2 receptor genes (122, 157, 158), which are associated with various autoimmune diseases, affects autoimmune disease development. In this regard, one would predict that a low IL-2 environment favors self-reactive Tfh cell differentiation and subsequent Auto-Ab production in autoimmune patients.

Synergistic Low-Dose rIL-2 Therapies

Recent studies suggest that in addition to secreting a low amount of IL-2, T cells from SLE patients poorly respond to exogenous IL-2 (159). Thus, the lack of IL-2 responsiveness could be a potential limitation when designing low-IL-2-based therapies to efficiently deplete Tfh cells *in vivo*. Importantly, data from our laboratory demonstrate that the IL-6/STAT3 pathway is an important regulator of the IL-2 responsiveness of Tfh cells. Briefly, using a combination of *in vivo* and genetic studies, we found that STAT3 in response to IL-6 binds to the *Il2rβ* locus and prevents CD122 up-regulation in Tfh cells, thereby limiting the capacity of these cells to respond to IL-2 (151). Hence, blockade of IL-6 signaling renders Tfh cells hyperresponsive to IL-2, thus lowering the threshold of IL-2 required to deplete Tfh cells (**Figure 3**). As a consequence, the frequency of Tfh cells was dramatically reduced in influenza-infected mice treated with an anti-IL-6 blockade in combination with rIL-2 compared to mice treated with rIL-2 alone, even when rIL-2 was administered at ultra-low doses.

These findings have important therapeutic and conceptual implications. In this regard, one would predict that *Tocilizumab*, a humanized anti-IL-6 receptor monoclonal antibody, administered together with rIL-2 will synergize to target the Tfh cell population more efficiently than rIL-2 administered alone. In this scenario, an IL-6 blockade would increase the expression of CD122 on the surface of Tfh cells, making them more susceptible to rIL-2 signaling and subsequent depletion. This combination therapy will likely achieve the same biological effect using a lower dose of rIL-2. In addition, IL-2 consumption by $CD25^+$Tregs limits the amount of available IL-2 (152, 160). Thus, it is likely that, in the presence of high numbers of CD25+Tregs, the environmental levels of IL-2 are scarce, and only cells with a relatively low response threshold will be able to respond to IL-2. Hence, another significant advantage of this approach is that, by increasing IL-2 responsiveness, this

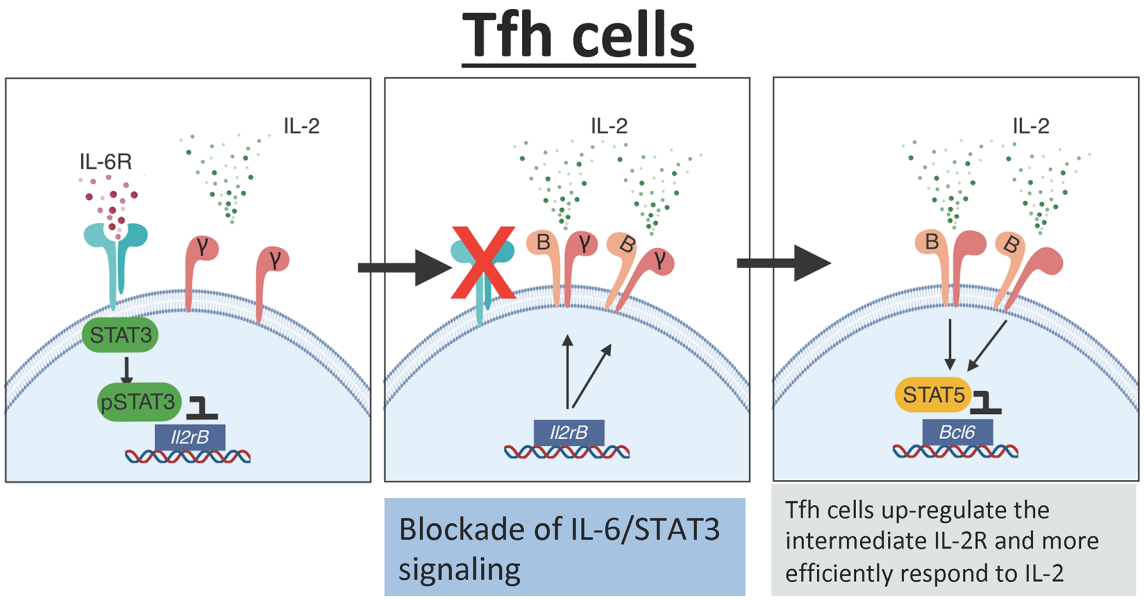


FIGURE 3 | IL-6 mediates the IL-2 responsiveness of Tfh cells. In the presence of IL-6 signaling, Tfh cells activate STAT3 which directly binds to the Il2rb promoter preventing expression of CD122. Low expression of CD122 limits IL-2 signaling in Tfh cells. In the absence of IL-6 signaling, there is increased expression of CD122 on the surface of Tfh cells leading to increased responsiveness to IL-2. The increased IL-2 signaling activates STAT5 which binds to the promoter region of Bcl6 and suppresses the Tfh cell program.

synergistic therapy will allow Tfh cells to react to rIL-2 even in the presence of IL-2-consuming Tregs, thereby simultaneously targeting Tregs and Tfh cells.

Importantly, similar to IL-6 (161, 162), the serum levels of the STAT3-activating cytokines IL-23 and IL-27 are increased in autoimmune patients (163–167). Thus, IL-6 signaling blockade alone might not be sufficient to enhance IL-2 responsiveness of Tfh cells due to the capacity of additional STAT3-activating cytokines to compensate for the lack of IL-6. Furthermore, IL-6, IL-23, and IL-27 all induce STAT3 activation *via* JAK2. In contrast, JAK2 is not required for IL-2 signaling. Notably, a recent study shows that the specific JAK2 inhibitor CEP-33779 can be safely administered to MRL.lpr mice, which show significant improvement in disease pathogenesis and reduced pSTAT3 levels after treatment (168). Given that JAK2 is required for STAT3 but not STAT5 activation, it is tempting to speculate that "*non-cytokine specific*" STAT3 inhibition after treatment with a JAK2 inhibitor will lower the threshold of IL-2 required for suppressing Tfh cells regardless of the presence of redundant STAT3-activating cytokines. In any case, altogether, these studies suggest a model in which STAT3 activation in response to STAT3-activating cytokines counterbalances IL-2-mediated suppression of Tfh cells by limiting IL-2 responsiveness of Tfh cells. A better knowledge of how the crosstalk between different cytokine pathways regulates Tfh cell development will allow us to design more efficient therapeutic strategies to prevent self-reactive Tfh cell responses in autoimmune patients, thereby precluding ensuing pathogenic B cells responses and Ab-mediated pathology.

IL-2 and Th17 Cells

The clinical benefits of targeting Th17 cells to prevent autoimmune manifestations have been explored in preclinical and clinical settings, and additional clinical trials are being conducted. The results, however, are conflicting (169–171). Independent randomized clinical trials demonstrate the clinical efficacy of targeting IL-17 to treat moderate to severe psoriasis. As a result, two monoclonal anti-IL-17A antibodies (secukinumab and ixekizumab) and one antibody targeting the IL-17 receptor (brodalumab) are now FDA approved for the treatment of this disease. However, the studies assessing the clinical benefits of anti-IL-17 biologics for the treatment of systemic rheumatologic disorders, such as RA or SLE, have yielded mixed results. While some preclinical studies and clinical trials show promising results after IL-17 blockade (169), the therapeutic effect of anti-IL-17 anti-IL-17A Abs is lower than anticipated.

The relatively low efficacy of these treatments is, to some extent, surprising, given the abundance of publications showing reduced pathology and severity after IL-17 blockade in preclinical animal models (172). One potential explanation for this discrepancy is patient sample heterogeneity. In this regard, while most of the studies show that elevated levels of IL-17 and high frequency of Th17 cells correlate with disease activity in SLE patients, some found no significant differences between patients and healthy controls (172). Since anti-IL-17 biological-based treatments will likely only be effective in patients with a "high IL-17" profile, the lack of proper patient stratification based on their IL-17 profile could explain the lack of consistency in the results. An alternative, but mutually complementary, explanation is the

inability of the current anti-IL-17 biologics to sufficiently block the aberrantly increased IL-17 pathway in these patients. Besides, IL-17/IL-17A blockade alone might not be sufficient to effectively disrupt the inflammatory cycle leading to disease pathogenesis once this has already been initiated.

As aforementioned, IL-2 signaling inhibits Th17 differentiation (106, 108, 110). As a consequence of this inhibitory effect, Th17 cells fail to differentiate in relatively high IL-2 environments. Contrariwise, IL-2 quenching facilitates Th17 cell development (109). Mechanistically, IL-2 antagonizes IL-17 differentiation *via* STAT5, which outcompetes STAT3 binding at the IL-17 locus, hence preventing binding of STAT3 and its enhancer elements in response to IL-6 (108). IL-2 signaling also represses IL-6R expression and recruits the histone deacetylator adaptor protein NCoR2 to the *Il17* locus, thereby contributing to further inhibiting IL-17 production (108). In agreement with the suppressor role of IL-2 in Th17 cell differentiation, the regulatory mechanisms that control IL-2 production also indirectly control IL-17 production. For example, the cAMP-responsive element modulator alpha (CREMα) negatively regulates IL-2 transcription by binding to the *Il2* locus (121, 173–175). IL-2 shortage after CREMα overexpression in T cells contributes to enhancing IL-17 differentiation, a phenomenon that can be reserved after IL-2 supplementation (175). Similarly, by suppressing IL-2 production, the phosphatase and tensin homologue (PTEN) indirectly favors Th17 differentiation (176). At a cellular level, IL-2 consumption by Treg cells favors Th17 development by creating a low IL-2 environment permissive for Th17 differentiation (160). Collectively, these studies demonstrate that Th17 cells preferably differentiate in "low-IL-2" environments.

Importantly, works from multiple laboratories demonstrate a causative relationship between IL-2 deficiency, subsequent excessive IL-17 responses, and autoimmune pathology development. For example, elegant work has shown that a lack of STAT3 activation prevents the accumulation of Th17 cells in IL-2-deficient mice, resulting in prolonged lifespan and reduced autoimmunity associated with IL-2 deficiency (108). Additional studies have shown that MRL/Fas(lpr/lpr) mice treated with rIL-2 have reduced frequency of IL-17 producing cells, which correlated with diminished disease manifestations (138). Moreover, in SLE patients, low-dose rIL-2 treatment resulted in reduced frequencies of Th17 cells, which correlated with the induction of remission in a recent open-labeled trial (143). Collectively, these results provide strong evidence for the therapeutic potential of rIL-2 to prevent unwanted Th17 responses *in vivo*. Importantly, current anti-IL-17 biologics target the product of Th17 cells (i.e., IL-17). In contrast, low-dose rIL-2 precludes the development of these cells, which has the potential to more effectively prevent IL-17-dependent immunopathology by preventing the continuous replenishment of Th17 cells from their precursors, thereby inducing long-lasting effects. Besides, anti-IL-17 biologics are limited in that their effect is restricted to limiting IL-17 responses. In contrast, rIL-2 therapy has broader effects beyond dampening IL-17, such as bolstering the Treg–mediated immunosuppression and/or decreasing autoreactive Tfh cells, which are likely to synergize with Th17 suppression to further prevent immunopathology. In this regard, given that Tfh and Th17 cells are similarly regulated by the IL-2/STA5 and IL-6/STAT3 pathways, the aforementioned combinational therapy with STAT3 blocking agents and rIL-2 is likely to simultaneously target Tfh and Th17 cells efficiently. In summary, the inhibitory effects of IL-2 in Th17 cells, and its subsequent effects on Auto-Ab responses and systemic inflammation, need to be evaluated when considering IL-2-based therapies for the treatment of autoimmune disorders.

IL-2 and DN T Cells

Despite the accumulating evidence supporting a pathogenic role for DN T cells, the exact mechanisms that regulate DN T cell homeostasis are unknown, and there are currently no therapies to selectively deplete DN T cells *in vivo*. Importantly, however, work from George Tsokos's group demonstrates that treatment with an inducible recombinant adeno-associated virus vector encoding IL-2 significantly reduced the frequency of IL-17$^+$ DN T cells in MRL/*lpr* mice, which was accompanied by reduced pathology and kidney infiltration (138). The effect of IL-2 on DN T cells is likely independent of the role of IL-2 in Treg cells, as treatment with IL-2 is complexed with the anti-IL-2 monoclonal JES6-1, which selectively target CD25-expressing Tregs did not affect DN T cells. Nevertheless, because DN T cells express neglectable levels of CD25 and CD122 and poorly phosphorylate STAT5 in response to IL-2, the authors suggest that the effect of IL-2 on DN T cells is indirect. In any case, whereas the exact mechanism by which IL-2 prevents DN T cell accumulation remains elusive, these studies demonstrate a critical role for IL-2 in preventing DN T cell expansion *in vivo*. Given the potential pathological role of these cells and their contribution to sustaining pathogenic Ab responses, the effects of low-dose rIL-2 immunotherapies on DN T cells should be carefully examined in future low-dose rIL-2 clinical trials.

CONCLUDING REMARKS

In conclusion, we present here the rationale for using new therapeutic regimens based on the combination of low-dose rIL-2 with other biologics to achieve ideal immunosuppression and improved disease scores. Since the original observation that in the absence of IL-2 signaling mice develop catastrophic autoimmune disease, our knowledge of the complex intersection of multiple underlining conditions contributing to autoimmunity has grown to include multiple T cell populations in addition to Treg cells. Armed with the understanding that Tfh, Th17, and DN T cells play critical roles in autoimmune disease progression and that they are efficiently depleted after rIL-2 treatment, it is time to consider how to leverage the broad-ranging effects of rIL-2 therapy to synergistically induce Treg cell immunosuppression along with the Tfh/Th17/DN T cells axis to efficiently prevent inflammation and auto-Ab-mediated pathology in autoimmune patients without the undesired side effects associated to systemic immunosuppression.

While some studies suggest that B cells do not express CD25 and STAT5 signaling is dispensable for B cell maturation and function (177), IL-2 favors B cell survival and PC differentiation

in vitro (178–180). These potential "*positive*" effects of IL-2 in B cells could, to some extent, compensate for the absence of T cell help and be detrimental in the context of B-cell mediated pathologies, particularly when administered for short periods of time. Therefore, though it is clear that low-dose rIL-2 therapies promote immunosuppression, the potential intrinsic effects of low-dose rIL-2 treatment in B cells should be carefully examined. Similarly, IL-2 inhibits the development of T follicular regulatory (TFR) cells (181–183), a particular subset of Tregs that express Bcl6 and CXCR5 and localize into the B cell follicles where they suppress Tfh and GC B cell responses (184–186). Mechanistically, IL-2 signaling induces Blimp-1 expression in conventional Tregs cells, thereby preventing them from up-regulating Bcl6 and becoming TFR cells. Given that TFR cells have a suppressive function in Tfh and GCs, the lack of these cells after low-dose rIL-2 could enhance pathogenic B cell responses. Corresponding with this idea, the absence of TFR cells favors the outgrowth of self-reactive B cell clones in some models (181, 185). Nevertheless, the role of TFR cells is more complex than initially expected, as, rather than inhibit, they promote GC and Ab responses in some models (187, 188). Besides, TFR cells express low levels of CD25 (181, 182). Thus, it is unlikely that rIL2 therapy will have a preferential impact on TFR cells. In any case, despite the putative adverse effects of IL-2, treatment with low-dose rIL-2 and IL-2/anti-IL-2 Ab complexes efficiently decreases anti-DNA Ab titers in NZB/W F1 mice (189) and hinders influenza-specific B cell responses in influenza-infected mice (114). These data support the view that, when used *in vivo,* the dominant effect of IL-2 in the B cell response is immunosuppression.

AUTHOR CONTRIBUTIONS

Both authors contributed equally. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by grants from the University of Alabama at Birmingham (UAB), the National Institutes of Health grant R01AI150664 to A. Ballesteros-Tato, and the Lupus Research Alliance Novel Research Award to A. Ballesteros-Tato.

ACKNOWLEDGMENTS

Figures were created with BioRender.com.

REFERENCES

1. Xiao ZX, Miller JS, Zheng SG. An Updated Advance of Autoantibodies in Autoimmune Diseases. *Autoimmun Rev* (2021) 20:102743. doi: 10.1016/j.autrev.2020.102743
2. Naparstek Y, Plotz PH. The Role of Autoantibodies in Autoimmune Disease. *Annu Rev Immunol* (1993) 11:79–104. doi: 10.1146/annurev.iy.11.040193.000455
3. Woodroffe AJ, Border WA, Theofilopoulos AN, Gotze O, Glassock RJ, Dixon FJ, et al. Detection of Circulating Immune Complexes in Patients With Glomerulonephritis. *Kidney Int* (1977) 12:268–78. doi: 10.1038/ki.1977.111
4. Greisman SG, Redecha PB, Kimberly RP, Christian CL. Differences Among Immune Complexes: Association of C1q in SLE Immune Complexes With Renal Disease. *J Immunol* (1987) 138:739–45.
5. Das A, Heesters BA, Bialas A, O'Flynn J, Rifkin IR, Ochando J, et al. Follicular Dendritic Cell Activation by TLR Ligands Promotes Autoreactive B Cell Responses. *Immunity* (2017) 46:106–19. doi: 10.1016/j.immuni.2016.12.014
6. Leslie D, Lipsky P, Notkins AL. Autoantibodies as Predictors of Disease. *J Clin Invest* (2001) 108:1417–22. doi: 10.1172/JCI14452
7. Nguyen DC, Joyner CJ, Sanz I, Lee FE. Factors Affecting Early Antibody Secreting Cell Maturation Into Long-Lived Plasma Cells. *Front Immunol* (2019) 10:2138. doi: 10.3389/fimmu.2019.02138
8. Radbruch A, Muehlinghaus G, Luger EO, Inamine A, Smith KG, Dorner T, et al. Competence and Competition: The Challenge of Becoming a Long-Lived Plasma Cell. *Nat Rev Immunol* (2006) 6:741–50. doi: 10.1038/nri1886
9. Bannard O, Cyster JG. Germinal Centers: Programmed for Affinity Maturation and Antibody Diversification. *Curr Opin Immunol* (2017) 45:21–30. doi: 10.1016/j.coi.2016.12.004
10. Malkiel S, Barlev AN, Atisha-Fregoso Y, Suurmond J, Diamond B. Plasma Cell Differentiation Pathways in Systemic Lupus Erythematosus. *Front Immunol* (2018) 9:427. doi: 10.3389/fimmu.2018.00427
11. 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
12. 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
13. Jenks SA, Cashman KS, Woodruff MC, Lee FE, Sanz I. Extrafollicular Responses in Humans and SLE. *Immunol Rev* (2019) 288:136–48. doi: 10.1111/imr.12741
14. Tipton CM, Fucile CF, Darce J, Chida A, Ichikawa T, Gregoretti I, et al. Diversity, Cellular Origin and Autoreactivity of Antibody-Secreting Cell Population Expansions in Acute Systemic Lupus Erythematosus. *Nat Immunol* (2015) 16:755–65. doi: 10.1038/ni.3175
15. Ramos-Casals M, Sanz I, Bosch X, Stone JH, Khamashta MA. B-Cell-Depleting Therapy in Systemic Lupus Erythematosus. *Am J Med* (2012) 125:327–36. doi: 10.1016/j.amjmed.2011.09.010
16. Lund FE, Randall TD. Effector and Regulatory B Cells: Modulators of CD4(+) T Cell Immunity. *Nat Rev Immunol* (2010) 10:236–47. doi: 10.1038/nri2729
17. Calero I, Sanz I. Targeting B Cells for the Treatment of SLE: The Beginning of the End or the End of the Beginning? *Discov Med* (2010) 10:416–24.
18. Cambridge G, Leandro MJ, Teodorescu M, Manson J, Rahman A, Isenberg DA, et al. B Cell Depletion Therapy in Systemic Lupus Erythematosus: Effect on Autoantibody and Antimicrobial Antibody Profiles. *Arthritis Rheum* (2006) 54:3612–22. doi: 10.1002/art.22211
19. Sanz I, Lee FE. B Cells as Therapeutic Targets in SLE. *Nat Rev Rheumatol* (2010) 6:326–37. doi: 10.1038/nrrheum.2010.68
20. Crotty S. Follicular Helper CD4 T Cells (TFH). *Annu Rev Immunol* (2011) 29:621–63. doi: 10.1146/annurev-immunol-031210-101400
21. 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
22. Goodnow CC, Vinuesa CG, Randall KL, Mackay F, Brink R. Control Systems and Decision Making for Antibody Production. *Nat Immunol* (2010) 11:681–8. doi: 10.1038/ni.1900
23. 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
24. 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-Fas(lpr/lpr)/J Mice. *J Immunol* (2012) 188:1656–67. doi: 10.4049/jimmunol.1003871
25. Crotty S. T Follicular Helper Cell Biology: A Decade of Discovery and Diseases. *Immunity* (2019) 50:1132–48. doi: 10.1016/j.immuni.2019.04.011
26. 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
27. 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
28. 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
29. 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.
30. 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) 207:353–63. doi: 10.1084/jem.20091738
31. 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
32. 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
33. 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
34. 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
35. 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
36. Mountz JD, Hsu HC, Ballesteros-Tato A. Dysregulation of T Follicular Helper Cells in Lupus. *J Immunol* (2019) 202:1649–58. doi: 10.4049/jimmunol.1801150
37. Gensous N, Charrier M, Duluc D, Contin-Bordes C, Truchetet ME, Lazaro E, et al. T Follicular Helper Cells in Autoimmune Disorders. *Front Immunol* (2018) 9:1637. doi: 10.3389/fimmu.2018.01637
38. 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
39. 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) 67:988–99. doi: 10.1002/art.39020
40. 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
41. 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
42. Bubier JA, Sproule TJ, Foreman O, Spolski R, Shaffer DJ, Morse HC3, 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 USA* (2009) 106:1518–23. doi: 10.1073/pnas.0807309106
43. 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) 182:1421–8. doi: 10.4049/jimmunol.182.3.1421
44. Sawaf M, Dumortier H, Monneaux F. Follicular Helper T Cells in Systemic Lupus Erythematosus: Why Should They Be Considered as Interesting Therapeutic Targets? *J Immunol Res* (2016) 2016:5767106. doi: 10.1155/2016/5767106
45. Weaver CT, Hatton RD, Mangan PR, Harrington LE. IL-17 Family Cytokines and the Expanding Diversity of Effector T Cell Lineages. *Annu Rev Immunol* (2007) 25:821–52. doi: 10.1146/annurev.immunol.25.022106.141557
46. Raymond W, Ostli-Eilertsen G, Griffiths S, Nossent J. IL-17A Levels in Systemic Lupus Erythematosus Associated With Inflammatory Markers and Lower Rates of Malignancy and Heart Damage: Evidence for a Dual Role. *Eur J Rheumatol* (2017) 4:29–35. doi: 10.5152/eurjrheum.2017.16059
47. Nalbandian A, Crispin JC, Tsokos GC. Interleukin-17 and Systemic Lupus Erythematosus: Current Concepts. *Clin Exp Immunol* (2009) 157:209–15. doi: 10.1111/j.1365-2249.2009.03944.x
48. 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
49. 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) 191:1614–24. doi: 10.4049/jimmunol.1300479
50. Tarlinton D. IL-17 Drives Germinal Center B Cells? *Nat Immunol* (2008) 9:124–6. doi: 10.1038/ni0208-124
51. Mitsdoerffer M, Lee Y, Jager A, Kim HJ, Korn T, Kolls JK, et al. Proinflammatory T Helper Type 17 Cells are Effective B-Cell Helpers. *Proc Natl Acad Sci USA* (2010) 107:14292–7. doi: 10.1073/pnas.1009234107
52. Patakas A, Benson RA, Withers DR, Conigliaro P, McInnes IB, Brewer JM, et al. Th17 Effector Cells Support B Cell Responses Outside of Germinal Centres. *PloS One* (2012) 7:e49715. doi: 10.1371/journal.pone.0049715
53. Amarilyo G, Lourenco EV, Shi FD, La Cava A. IL-17 Promotes Murine Lupus. *J Immunol* (2014) 193:540–3. doi: 10.4049/jimmunol.1400931
54. Kyttaris VC, Zhang Z, Kuchroo VK, Oukka M, Tsokos GC. Cutting Edge: IL-23 Receptor Deficiency Prevents the Development of Lupus Nephritis in C57BL/6-lpr/lpr Mice. *J Immunol* (2010) 184:4605–9. doi: 10.4049/jimmunol.0903595
55. Dai H, He F, Tsokos GC, Kyttaris VC. IL-23 Limits the Production of IL-2 and Promotes Autoimmunity in Lupus. *J Immunol* (2017) 199:903–10. doi: 10.4049/jimmunol.1700418
56. Wei L, Laurence A, Elias KM, O'Shea JJ. IL-21 is Produced by Th17 Cells and Drives IL-17 Production in a STAT3-Dependent Manner. *J Biol Chem* (2007) 282:34605–10. doi: 10.1074/jbc.M705100200
57. Doreau A, Belot A, Bastid J, Riche B, Trescol-Biemont MC, Ranchin B, et al. Interleukin 17 Acts in Synergy With B Cell-Activating Factor to Influence B Cell Biology and the Pathophysiology of Systemic Lupus Erythematosus. *Nat Immunol* (2009) 10:778–85. doi: 10.1038/ni.1741
58. Flannigan KL, Ngo VL, Geem D, Harusato A, Hirota SA, Parkos CA, et al. IL-17A-Mediated Neutrophil Recruitment Limits Expansion of Segmented Filamentous Bacteria. *Mucosal Immunol* (2017) 10:673–84. doi: 10.1038/mi.2016.80
59. Ye P, Rodriguez FH, Kanaly S, Stocking KL, Schurr J, Schwarzenberger P, et al. Requirement of Interleukin 17 Receptor Signaling for Lung CXC Chemokine and Granulocyte Colony-Stimulating Factor Expression, Neutrophil Recruitment, and Host Defense. *J Exp Med* (2001) 194:519–27. doi: 10.1084/jem.194.4.519
60. Parsa R, Lund H, Georgoudaki AM, Zhang XM, Ortlieb Guerreiro-Cacais A, Grommisch D, et al. BAFF-Secreting Neutrophils Drive Plasma Cell Responses During Emergency Granulopoiesis. *J Exp Med* (2016) 213:1537–53. doi: 10.1084/jem.20150577
61. Cerutti A, Puga I, Magri G. The B Cell Helper Side of Neutrophils. *J Leukoc Biol* (2013) 94:677–82. doi: 10.1189/jlb.1112596
62. 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
63. 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

64. 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
65. 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
66. Paulos CM, Carpenito C, Plesa G, Suhoski MM, Varela-Rohena A, Golovina TN, et al. The Inducible Costimulator (ICOS) is Critical for the Development of Human T(H)17 Cells. *Sci Transl Med* (2010) 2:55ra78. doi: 10.1126/scitranslmed.3000448
67. Korn T, Mitsdoerffer M, Croxford AL, Awasthi A, Dardalhon VA, Galileos G, et al. IL-6 Controls Th17 Immunity in Vivo by Inhibiting the Conversion of Conventional T Cells Into Foxp3+ Regulatory T Cells. *Proc Natl Acad Sci USA* (2008) 105:18460–5. doi: 10.1073/pnas.0809850105
68. Mondal A, Sawant D, Dent AL. Transcriptional Repressor BCL6 Controls Th17 Responses by Controlling Gene Expression in Both T Cells and Macrophages. *J Immunol* (2010) 184:4123–32. doi: 10.4049/jimmunol.0901242
69. 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
70. 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
71. Ballesteros-Tato A, Randall TD, Lund FE, Spolski R, Leonard WJ, Leon 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
72. Gowthaman U, Chen JS, Zhang B, Flynn WF, Lu Y, Song W, et al. Identification of a T Follicular Helper Cell Subset That Drives Anaphylactic Ige. *Science* (2019) 365:eaaw6433. doi: 10.1126/science.aaw6433
73. 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
74. Trub 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
75. 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
76. D'Acquisto F, Crompton T. CD3+CD4-CD8- (Double Negative) T Cells: Saviours or Villains of the Immune Response? *Biochem Pharmacol* (2011) 82:333–40. doi: 10.1016/j.bcp.2011.05.019
77. Martina MN, Noel S, Saxena A, Rabb H, Hamad AR. Double Negative (DN) Alphabeta T Cells: Misperception and Overdue Recognition. *Immunol Cell Biol* (2015) 93:305–10. doi: 10.1038/icb.2014.99
78. Tarbox JA, Keppel MP, Topcagic N, Mackin C, Ben Abdallah M, Baszis KW, et al. Elevated Double Negative T Cells in Pediatric Autoimmunity. *J Clin Immunol* (2014) 34:594–9. doi: 10.1007/s10875-014-0038-z
79. Lim MS, Straus SE, Dale JK, Fleisher TA, Stetler-Stevenson M, Strober W, et al. Pathological Findings in Human Autoimmune Lymphoproliferative Syndrome. *Am J Pathol* (1998) 153:1541–50. doi: 10.1016/S0002-9440(10)65742-2
80. Price S, Shaw PA, Seitz A, Joshi G, Davis J, Niemela JE, et al. Natural History of Autoimmune Lymphoproliferative Syndrome Associated With FAS Gene Mutations. *Blood* (2014) 123:1989–99. doi: 10.1182/blood-2013-10-535393
81. Laouar Y, Ezine S. In Vivo CD4+ Lymph Node T Cells From Lpr Mice Generate CD4-CD8-B220+TCR-Beta Low Cells. *J Immunol* (1994) 153:3948–55.
82. Crispin JC, Tsokos GC. Human TCR-Alpha Beta+ CD4- CD8- T Cells Can Derive From CD8+ T Cells and Display an Inflammatory Effector Phenotype. *J Immunol* (2009) 183:4675–81. doi: 10.4049/jimmunol.0901533
83. Li H, Adamopoulos IE, Moulton VR, Stillman IE, Herbert Z, Moon JJ, et al. Systemic Lupus Erythematosus Favors the Generation of IL-17 Producing Double Negative T Cells. *Nat Commun* (2020) 11:2859. doi: 10.1038/s41467-020-16636-4
84. Masuda T, Ohteki T, Abo T, Seki S, Nose S, Nagura H, et al. Expansion of the Population of Double Negative CD4-8- T Alpha Beta-Cells in the Liver is a Common Feature of Autoimmune Mice. *J Immunol* (1991) 9:2907–12, 147.
85. Shivakumar S, Tsokos GC, Datta SK. T Cell Receptor Alpha/Beta Expressing Double-Negative (CD4-/CD8-) and CD4+ T Helper Cells in Humans Augment the Production of Pathogenic Anti-DNA Autoantibodies Associated With Lupus Nephritis. *J Immunol* (1989) 143:103–12.
86. 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
87. Sieling PA, Porcelli SA, Duong BT, Spada F, Bloom BR, Diamond B, et al. Human Double-Negative T Cells in Systemic Lupus Erythematosus Provide Help for IgG and are Restricted by CD1c. *J Immunol* (2000) 165:5338–44. doi: 10.4049/jimmunol.165.9.5338
88. Crispin JC, Oukka M, Bayliss G, Cohen RA, Van Beek CA, Stillman IE, et al. Expanded Double Negative T Cells in Patients With Systemic Lupus Erythematosus Produce IL-17 and Infiltrate the Kidneys. *J Immunol* (2008) 181:8761–6. doi: 10.4049/jimmunol.181.12.8761
89. Liu SM, King C. IL-21-Producing Th Cells in Immunity and Autoimmunity. *J Immunol* (2013) 191:3501–6. doi: 10.4049/jimmunol.1301454
90. Morgan DA, Ruscetti FW, Gallo R. Selective in Vitro Growth of T Lymphocytes From Normal Human Bone Marrows. *Science* (1976) 193:1007–8. doi: 10.1126/science.181845
91. Ruscetti FW, Morgan DA, Gallo RC. Functional and Morphologic Characterization of Human T Cells Continuously Grown in Vitro. *J Immunol* (1977) 119:131–8.
92. Mier JW, Gallo RC. Purification and Some Characteristics of Human T-Cell Growth Factor From Phytohemagglutinin-Stimulated Lymphocyte-Conditioned Media. *Proc Natl Acad Sci USA* (1980) 77:6134–8. doi: 10.1073/pnas.77.10.6134
93. Bayer AL, Pugliese A, Malek TR. The IL-2/IL-2R System: From Basic Science to Therapeutic Applications to Enhance Immune Regulation. *Immunol Res* (2013) 57:197–209. doi: 10.1007/s12026-013-8452-5
94. Wang X, Rickert M, Garcia KC. Structure of the Quaternary Complex of Interleukin-2 With Its Alpha, Beta, and Gammac Receptors. *Science* (2005) 310:1159–63. doi: 10.1126/science.1117893
95. Rickert M, Wang X, Boulanger MJ, Goriatcheva N, Garcia KC. The Structure of Interleukin-2 Complexed With Its Alpha Receptor. *Science* (2005) 308:1477–80. doi: 10.1126/science.1109745
96. Malek TR, Yu A, Vincek V, Scibelli P, Kong L. CD4 Regulatory T Cells Prevent Lethal Autoimmunity in IL-2Rbeta-Deficient Mice. Implications for the Nonredundant Function of IL-2. *Immunity* (2002) 17:167–78. doi: 10.1016/S1074-7613(02)00367-9
97. Rudensky AY. Regulatory T Cells and Foxp3. *Immunol Rev* (2011) 241:260–8. doi: 10.1111/j.1600-065X.2011.01018.x
98. Fontenot JD, Rasmussen JP, Gavin MA, Rudensky AY. A Function for Interleukin 2 in Foxp3-Expressing Regulatory T Cells. *Nat Immunol* (2005) 6:1142–51. doi: 10.1038/ni1263
99. Yuan X, Cheng G, Malek TR. The Importance of Regulatory T-Cell Heterogeneity in Maintaining Self-Tolerance. *Immunol Rev* (2014) 259:103–14. doi: 10.1111/imr.12163
100. Ballesteros-Tato A. Beyond regulatory T cells: the potential role for IL-2 to deplete T-follicular helper cells and treat autoimmune diseases. *Immunotherapy* (2014) 6(11):1207–20. doi: 10.2217/imt.14.83
101. Malek TR. The Biology of Interleukin-2. *Annu Rev Immunol* (2008) 26:453–79. doi: 10.1146/annurev.immunol.26.021607.090357
102. Zorn E, Nelson EA, Mohseni M, Porcheray F, Kim H, Litsa D, et al. IL-2 Regulates FOXP3 Expression in Human CD4+CD25+ Regulatory T Cells Through a STAT-Dependent Mechanism and Induces the Expansion of These Cells in Vivo. *Blood* (2006) 108:1571–9. doi: 10.1182/blood-2006-02-004747
103. Murawski MR, Litherland SA, Clare-Salzler MJ, Davoodi-Semiromi A. Upregulation of Foxp3 Expression in Mouse and Human Treg is IL-2/STAT5 Dependent: Implications for the NOD STAT5B Mutation in Diabetes Pathogenesis. *Ann N Y Acad Sci* (2006) 1079:198–204. doi: 10.1196/annals.1375.031

104. Passerini L, Allan SE, Battaglia M, Di Nunzio S, Alstad AN, Levings MK, et al. STAT5-Signaling Cytokines Regulate the Expression of FOXP3 in CD4 +CD25+ Regulatory T Cells and CD4+CD25- Effector T Cells. *Int Immunol* (2008) 20:421–31. doi: 10.1093/intimm/dxn002
105. Pipkin ME, Sacks JA, Cruz-Guilloty F, Lichtenheld MG, Bevan MJ, Rao A. Interleukin-2 and Inflammation Induce Distinct Transcriptional Programs That Promote the Differentiation of Effector Cytolytic T Cells. *Immunity* (2010) 32:79–90. doi: 10.1016/j.immuni.2009.11.012
106. 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
107. 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
108. Yang XP, Ghoreschi K, Steward-Tharp SM, Rodriguez-Canales J, Zhu J, Grainger JR, et al. Opposing Regulation of the Locus Encoding IL-17 Through Direct, Reciprocal Actions of STAT3 and STAT5. *Nat Immunol* (2011) 12:247–54. doi: 10.1038/ni.1995
109. Chen Y, Haines CJ, Gutcher I, Hochweller K, Blumenschein WM, McClanahan T, et al. Foxp3(+) Regulatory T Cells Promote T Helper 17 Cell Development in Vivo Through Regulation of Interleukin-2. *Immunity* (2011) 34:409–21. doi: 10.1016/j.immuni.2011.02.011
110. Laurence A, Tato CM, Davidson TS, Kanno Y, Chen Z, Yao Z, et al. Interleukin-2 Signaling Via STAT5 Constrains T Helper 17 Cell Generation. *Immunity* (2007) 26:371–81. doi: 10.1016/j.immuni.2007.02.009
111. 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
112. Walker SR, Nelson EA, Frank DA. STAT5 Represses BCL6 Expression by Binding to a Regulatory Region Frequently Mutated in Lymphomas. *Oncogene* (2007) 26:224–33. doi: 10.1038/sj.onc.1209775
113. 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
114. 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
115. Suzuki H, Duncan GS, Takimoto H, Mak TW. Abnormal Development of Intestinal Intraepithelial Lymphocytes and Peripheral Natural Killer Cells in Mice Lacking the IL-2 Receptor Beta Chain. *J Exp Med* (1997) 185:499–505. doi: 10.1084/jem.185.3.499
116. Sadlack B, Lohler J, Schorle H, Klebb G, Haber H, Sickel E, et al. Generalized Autoimmune Disease in Interleukin-2-Deficient Mice is Triggered by an Uncontrolled Activation and Proliferation of CD4+ T Cells. *Eur J Immunol* (1995) 25:3053–9. doi: 10.1002/eji.1830251111
117. Suzuki H, Kundig TM, Furlonger C, Wakeham A, Timms E, Matsuyama T, et al. Deregulated T Cell Activation and Autoimmunity in Mice Lacking Interleukin-2 Receptor Beta. *Science* (1995) 268:1472–6. doi: 10.1126/science.7770771
118. Sadlack B, Merz H, Schorle H, Schimpl A, Feller AC, Horak I. Ulcerative Colitis-Like Disease in Mice With a Disrupted Interleukin-2 Gene. *Cell* (1993) 75:253–61. doi: 10.1016/0092-8674(93)80067-O
119. Ballesteros-Tato A, Papillion A. Mechanisms of Action of Low-Dose IL-2 Restoration Therapies in SLE. *Curr Opin Immunol* (2019) 61:39–45. doi: 10.1016/j.coi.2019.07.003
120. Lieberman LA, Tsokos GC. The IL-2 Defect in Systemic Lupus Erythematosus Disease Has an Expansive Effect on Host Immunity. *J BioMed Biotechnol* (2010) 2010:740619. doi: 10.1155/2010/740619
121. Solomou EE, Juang YT, Gourley MF, Kammer GM, Tsokos GC. Molecular Basis of Deficient IL-2 Production in T Cells From Patients With Systemic Lupus Erythematosus. *J Immunol* (2001) 166:4216–22. doi: 10.4049/jimmunol.166.6.4216
122. Yamanouchi J, Rainbow D, Serra P, Howlett S, Hunter K, Garner VE, et al. Interleukin-2 Gene Variation Impairs Regulatory T Cell Function and Causes Autoimmunity. *Nat Genet* (2007) 39:329–37. doi: 10.1038/ng1958
123. Linker-Israeli M, Bakke AC, Kitridou RC, Gendler S, Gillis S, Horwitz DA. Defective Production of Interleukin 1 and Interleukin 2 in Patients With Systemic Lupus Erythematosus (SLE). *J Immunol* (1983) 130:2651–5.
124. 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
125. Brandenburg S, Takahashi T, de la Rosa M, Janke M, Karsten G, Muzzulini T, et al. IL-2 Induces in Vivo Suppression by CD4(+)CD25(+)Foxp3(+) Regulatory T Cells. *Eur J Immunol* (2008) 38:1643–53. doi: 10.1002/eji.200737791
126. Furtado GC, Curotto de Lafaille MA, Kutchukhidze N, Lafaille JJ. Interleukin 2 Signaling is Required for CD4(+) Regulatory T Cell Function. *J Exp Med* (2002) 196:851–7. doi: 10.1084/jem.20020190
127. Kim JM, Rasmussen JP, Rudensky AY. Regulatory T Cells Prevent Catastrophic Autoimmunity Throughout the Lifespan of Mice. *Nat Immunol* (2007) 8:191–7. doi: 10.1038/ni1428
128. Brunkow ME, Jeffery EW, Hjerrild KA, Paeper B, Clark LB, Yasayko SA, et al. Disruption of a New Forkhead/Winged-Helix Protein, Scurfin, Results in the Fatal Lymphoproliferative Disorder of the Scurfy Mouse. *Nat Genet* (2001) 27:68–73. doi: 10.1038/83784
129. Bennett CL, Christie J, Ramsdell F, Brunkow ME, Ferguson PJ, Whitesell L, 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
130. 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
131. Tang Q, Adams JY, Penaranda C, Melli K, Piaggio E, Sgouroudis E, et al. Central Role of Defective Interleukin-2 Production in the Triggering of Islet Autoimmune Destruction. *Immunity* (2008) 28:687–97. doi: 10.1016/j.immuni.2008.03.016
132. Diaz-de-Durana Y, Lau J, Knee D, Filippi C, Londei M, McNamara P, et al. IL-2 Immunotherapy Reveals Potential for Innate Beta Cell Regeneration in the Non-Obese Diabetic Mouse Model of Autoimmune Diabetes. *PloS One* (2013) 8:e78483. doi: 10.1371/journal.pone.0078483
133. Grinberg-Bleyer Y, Baeyens A, You S, Elhage R, Fourcade G, Gregoire S, et al. IL-2 Reverses Established Type 1 Diabetes in NOD Mice by a Local Effect on Pancreatic Regulatory T Cells. *J Exp Med* (2010) 207:1871–8. doi: 10.1084/jem.20100209
134. Webster KE, Walters S, Kohler RE, Mrkvan T, Boyman O, Surh CD, et al. In Vivo Expansion of T Reg Cells With IL-2-mAb Complexes: Induction of Resistance to EAE and Long-Term Acceptance of Islet Allografts Without Immunosuppression. *J Exp Med* (2009) 206:751–60. doi: 10.1084/jem.20082824
135. Liu R, Zhou Q, La Cava A, Campagnolo DI, Van Kaer L, Shi FD. Expansion of Regulatory T Cells Via IL-2/Anti-IL-2 mAb Complexes Suppresses Experimental Myasthenia. *Eur J Immunol* (2010) 40:1577–89. doi: 10.1002/eji.200939792
136. Shapiro AM, Suarez-Pinzon WL, Power R, Rabinovitch A. Combination Therapy With Low Dose Sirolimus and Tacrolimus is Synergistic in Preventing Spontaneous and Recurrent Autoimmune Diabetes in Non-Obese Diabetic Mice. *Diabetologia* (2002) 45:224–30. doi: 10.1007/s00125-001-0745-x
137. Baeyens A, Perol L, Fourcade G, Cagnard N, Carpentier W, Woytschak J, et al. Limitations of IL-2 and Rapamycin in Immunotherapy of Type 1 Diabetes. *Diabetes* (2013) 62:3120–31. doi: 10.2337/db13-0214
138. Mizui M, Koga T, Lieberman LA, Beltran J, Yoshida N, Johnson MC, et al. IL-2 Protects Lupus-Prone Mice From Multiple End-Organ Damage by Limiting CD4-CD8- IL-17-Producing T Cells. *J Immunol* (2014) 193:2168–77. doi: 10.4049/jimmunol.1400977
139. 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
140. Hartemann A, Bensimon G, Payan CA, Jacqueminet S, Bourron O, Nicolas N, et al. Low-Dose Interleukin 2 in Patients With Type 1 Diabetes: A Phase 1/2 Randomised, Double-Blind, Placebo-Controlled Trial. *Lancet Diabetes Endocrinol* (2013) 1:295–305. doi: 10.1016/S2213-8587(13)70113-X

141. Saadoun D, Rosenzwajg M, Joly F, Six A, Carrat F, Thibault V, et al. Regulatory T-Cell Responses to Low-Dose Interleukin-2 in HCV-Induced Vasculitis. *N Engl J Med* (2011) 365:2067–77. doi: 10.1056/NEJMoa1105143
142. He J, Zhang R, Shao M, Zhao X, Miao M, Chen J, et al. Efficacy and Safety of Low-Dose IL-2 in the Treatment of Systemic Lupus Erythematosus: A Randomised, Double-Blind, Placebo-Controlled Trial. *Ann Rheum Dis* (2020) 79:141–9. doi: 10.1136/annrheumdis-2019-215396
143. 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
144. Humrich JY, Riemekasten G. Low-Dose Interleukin-2 Therapy for the Treatment of Systemic Lupus Erythematosus. *Curr Opin Rheumatol* (2019) 31:208–12. doi: 10.1097/BOR.0000000000000575
145. Humrich JY, von Spee-Mayer C, Siegert E, Alexander T, Hiepe F, Radbruch A, et al. Rapid Induction of Clinical Remission by Low-Dose Interleukin-2 in a Patient With Refractory SLE. *Ann Rheum Dis* (2015) 74:791–2. doi: 10.1136/annrheumdis-2014-206506
146. Matsuoka K, Koreth J, Kim HT, Bascug G, McDonough S, Kawano Y, et al. Low-Dose Interleukin-2 Therapy Restores Regulatory T Cell Homeostasis in Patients With Chronic Graft-Versus-Host Disease. *Sci Transl Med* (2013) 5:179ra43. doi: 10.1126/scitranslmed.3005265
147. Kennedy-Nasser AA, Ku S, Castillo-Caro P, Hazrat Y, Wu MF, Liu H, et al. Ultra Low-Dose IL-2 for GVHD Prophylaxis After Allogeneic Hematopoietic Stem Cell Transplantation Mediates Expansion of Regulatory T Cells Without Diminishing Antiviral and Antileukemic Activity. *Clin Cancer Res* (2014) 20:2215–25. doi: 10.1158/1078-0432.CCR-13-3205
148. von Spee-Mayer C, Siegert E, Abdirama D, Rose A, Klaus A, Alexander T, et al. Low-Dose Interleukin-2 Selectively Corrects Regulatory T Cell Defects in Patients With Systemic Lupus Erythematosus. *Ann Rheum Dis* (2016) 75:1407–15. doi: 10.1136/annrheumdis-2015-207776
149. Rosenzwajg M, Lorenzon R, Cacoub P, Pham HP, Pitoiset F, El Soufi K, et al. Immunological and Clinical Effects of Low-Dose Interleukin-2 Across 11 Autoimmune Diseases in a Single, Open Clinical Trial. *Ann Rheum Dis* (2019) 78:209–17. doi: 10.1136/annrheumdis-2018-214229
150. Shevach EM. Application of IL-2 Therapy to Target T Regulatory Cell Function. *Trends Immunol* (2012) 33:626–32. doi: 10.1016/j.it.2012.07.007
151. Papillion A, Powell MD, Chisolm DA, Bachus H, Fuller MJ, Weinmann AS, et al. Inhibition of IL-2 Responsiveness by IL-6 is Required for the Generation of GC-TFH Cells. *Sci Immunol* (2019) 4:eaaw7636. doi: 10.1126/sciimmunol.aaw7636
152. 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
153. 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
154. Schmitt N, Liu Y, Bentebibel SE, Ueno H. Molecular Mechanisms Regulating T Helper 1 Versus T Follicular Helper Cell Differentiation in Humans. *Cell Rep* (2016) 16:1082–95. doi: 10.1016/j.celrep.2016.06.063
155. 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
156. 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
157. Brand OJ, Lowe CE, Heward JM, Franklyn JA, Cooper JD, Todd JA, et al. Association of the Interleukin-2 Receptor Alpha (IL-2Ralpha)/CD25 Gene Region With Graves' Disease Using a Multilocus Test and Tag SNPs. *Clin Endocrinol (Oxf)* (2007) 66:508–12. doi: 10.1111/j.1365-2265.2007.02762.x
158. Matesanz F, Fedetz M, Collado-Romero M, Fernandez O, Guerrero M, Delgado C, et al. Allelic Expression and Interleukin-2 Polymorphisms in Multiple Sclerosis. *J Neuroimmunol* (2001) 119:101–5. doi: 10.1016/S0165-5728(01)00354-X
159. Comte D, Karampetsou MP, Kis-Toth K, Yoshida N, Bradley SJ, Kyttaris VC, et al. Brief Report: CD4+ T Cells From Patients With Systemic Lupus Erythematosus Respond Poorly to Exogenous Interleukin-2. *Arthritis Rheumatol* (2017) 69:808–13. doi: 10.1002/art.40014
160. Pandiyan P, Conti HR, Zheng L, Peterson AC, Mathern DR, Hernandez-Santos N, et al. CD4(+)CD25(+)Foxp3(+) Regulatory T Cells Promote Th17 Cells in Vitro and Enhance Host Resistance in Mouse Candida Albicans Th17 Cell Infection Model. *Immunity* (2011) 34:422–34. doi: 10.1016/j.immuni.2011.03.002
161. Tackey E, Lipsky PE, Illei GG. Rationale for Interleukin-6 Blockade in Systemic Lupus Erythematosus. *Lupus* (2004) 13:339–43. doi: 10.1191/0961203304lu1023oa
162. Linker-Israeli M, Deans RJ, Wallace DJ, Prehn J, Ozeri-Chen T, Klinenberg JR. Elevated Levels of Endogenous IL-6 in Systemic Lupus Erythematosus. a Putative Role in Pathogenesis. *J Immunol* (1991) 147:117–23.
163. Wong CK, Lit LC, Tam LS, Li EK, Wong PT, Lam CW. Hyperproduction of IL-23 and IL-17 in Patients With Systemic Lupus Erythematosus: Implications for Th17-Mediated Inflammation in Auto-Immunity. *Clin Immunol* (2008) 127:385–93. doi: 10.1016/j.clim.2008.01.019
164. Du J, Li Z, Shi J, Bi L. Associations Between Serum Interleukin-23 Levels and Clinical Characteristics in Patients With Systemic Lupus Erythematosus. *J Int Med Res* (2014) 42:1123–30. doi: 10.1177/0300060513509130
165. Leng RX, Pan HF, Chen GM, Wang C, Qin WZ, Chen LL, et al. IL-23: A Promising Therapeutic Target for Systemic Lupus Erythematosus. *Arch Med Res* (2010) 41:221–5. doi: 10.1016/j.arcmed.2010.02.011
166. Qiu F, Song L, Yang N, Li X. Glucocorticoid Downregulates Expression of IL-12 Family Cytokines in Systemic Lupus Erythematosus Patients. *Lupus* (2013) 22:1011–6. doi: 10.1177/0961203313498799
167. Xia LP, Li BF, Shen H, Lu J. Interleukin-27 and Interleukin-23 in Patients With Systemic Lupus Erythematosus: Possible Role in Lupus Nephritis. *Scand J Rheumatol* (2015) 44:200–5. doi: 10.3109/03009742.2014.962080
168. Lu LD, Stump KL, Wallace NH, Dobrzanski P, Serdikoff C, Gingrich DE, et al. Depletion of Autoreactive Plasma Cells and Treatment of Lupus Nephritis in Mice Using CEP-33779, a Novel, Orally Active, Selective Inhibitor of JAK2. *J Immunol* (2011) 187:3840–53. doi: 10.4049/jimmunol.1101228
169. Taams LS. Interleukin-17 in Rheumatoid Arthritis: Trials and Tribulations. *J Exp Med* (2020) 217:3840–53. doi: 10.1084/jem.20192048
170. Yang J, Sundrud MS, Skepner J, Yamagata T. Targeting Th17 Cells in Autoimmune Diseases. *Trends Pharmacol Sci* (2014) 35:493–500. doi: 10.1016/j.tips.2014.07.006
171. McGeachy MJ, Cua DJ, Gaffen SL. The IL-17 Family of Cytokines in Health and Disease. *Immunity* (2019) 50:892–906. doi: 10.1016/j.immuni.2019.03.021
172. Robert M, Miossec P. Interleukin-17 and Lupus: Enough to Be a Target? for Which Patients? *Lupus* (2020) 29:6–14. doi: 10.1177/0961203319891243
173. Juang YT, Wang Y, Solomou EE, Li Y, Mawrin C, Tenbrock K, et al. Systemic Lupus Erythematosus Serum IgG Increases CREM Binding to the IL-2 Promoter and Suppresses IL-2 Production Through CaMKIV. *J Clin Invest* (2005) 115:996–1005. doi: 10.1172/JCI22854
174. Tenbrock K, Juang YT, Gourley MF, Nambiar MP, Tsokos GC. Antisense Cyclic Adenosine 5'-Monophosphate Response Element Modulator Up-Regulates IL-2 in T Cells From Patients With Systemic Lupus Erythematosus. *J Immunol* (2002) 169:4147–52. doi: 10.4049/jimmunol.169.8.4147
175. Lippe R, Ohl K, Varga G, Rauen T, Crispin JC, Juang YT, et al. Cremalpha Overexpression Decreases IL-2 Production, Induces a T(H)17 Phenotype and Accelerates Autoimmunity. *J Mol Cell Biol* (2012) 4:121–3. doi: 10.1093/jmcb/mjs004
176. Kim HS, Jang SW, Lee W, Kim K, Sohn H, Hwang SS, et al. PTEN Drives Th17 Cell Differentiation by Preventing IL-2 Production. *J Exp Med* (2017) 214:3381–98. doi: 10.1084/jem.20170523
177. Dai X, Chen Y, Di L, Podd A, Li G, Bunting KD, et al. Stat5 is Essential for Early B Cell Development But Not for B Cell Maturation and Function. *J Immunol* (2007) 179:1068–79. doi: 10.4049/jimmunol.179.2.1068
178. Mingari MC, Gerosa F, Carra G, Accolla RS, Moretta A, Zubler RH, et al. Human Interleukin-2 Promotes Proliferation of Activated B Cells Via Surface Receptors Similar to Those of Activated T Cells. *Nature* (1984) 312:641–3. doi: 10.1038/312641a0
179. Hipp N, Symington H, Pastoret C, Caron G, Monvoisin C, Tarte K, et al. IL-2 Imprints Human Naive B Cell Fate Towards Plasma Cell Through ERK/

ELK1-Mediated BACH2 Repression. *Nat Commun* (2017) 8:1443. doi: 10.1038/s41467-017-01475-7
180. Le Gallou S, Caron G, Delaloy C, Rossille D, Tarte K, Fest T. IL-2 Requirement for Human Plasma Cell Generation: Coupling Differentiation and Proliferation by Enhancing MAPK-ERK Signaling. *J Immunol* (2012) 189:161–73. doi: 10.4049/jimmunol.1200301
181. 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
182. 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
183. Fonseca VR, Ribeiro F, Graca L. T Follicular Regulatory (Tfr) Cells: Dissecting the Complexity of Tfr-Cell Compartments. *Immunol Rev* (2019) 288:112–27. doi: 10.1111/imr.12739
184. 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
185. 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
186. 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
187. 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
188. 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
189. Yan JJ, Lee JG, Jang JY, Koo TY, Ahn C, Yang J. IL-2/Anti-IL-2 Complexes Ameliorate Lupus Nephritis by Expansion of CD4(+)CD25(+)Foxp3(+) Regulatory T Cells. *Kidney Int* (2017) 91:603–15. doi: 10.1016/j.kint.2016.09.022

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.

frontiers in Immunology

ORIGINAL RESEARCH
published: 19 May 2021
doi: 10.3389/fimmu.2021.662901

Effects of Peptide-Induced Immune Tolerance on Murine Lupus

Ram P. Singh[1,2*†], *Bevra H. Hahn*[2] *and David S. Bischoff*[1,3]

[1] Research Service, Veteran Administration Greater Los Angeles Healthcare System, Los Angeles, CA, United States,
[2] Division of Rheumatology, Department of Medicine, University of California, Los Angeles, Los Angeles, CA, United States,
[3] Department of Medicine, University of California, Los Angeles, Los Angeles, CA, United States

The regulation of autoimmunity and the molecular mechanisms by which different immune cells, including T cells, polymorphonuclear leukocytes (PMN-granulocytes), and B cells suppress autoimmune diseases is complex. We have shown previously that BWF1 lupus mice are protected from autoimmunity after *i.v.* injection or oral administration of tolerogenic doses of pCons, an artificial synthetic peptide based on sequences containing MHC class I and MHC class II determinants in the VH region of a J558-encoded BWF1 anti-DNA Ab. Several T cell subsets can transfer this tolerance. In this study, we determined the potential roles of granulocytes, B cells and regulatory T cells altered by pCons treatment in the BWF1 (NZB/NZW) mouse model of lupus. Immunophenotyping studies indicated that pCons treatment of BWF1 mice significantly increased $CD4^+FoxP3^+$ T cells, reduced the percent of B cells expressing $CD19^+CD5^+$ but increased the percent of $CD19^+CD1d^+$ regulatory B cells and increased the ability of the whole B cell population to suppress IgG anti-DNA production *in vitro*. pCons treatment significantly decreased the expression of CTLA-4 (cytotoxic T-lymphocyte-associated protein-4) in $CD8^+$ T cells. In addition, peptide administration modified granulocytes so they became suppressive. We co-cultured sorted naïve B cells from mice making anti-DNA Ab (supported by addition of sorted naive $CD4^+$ and $CD8^+$ T cells from young auto-antibody-negative BWF1 mice) with sorted B cells or granulocytes from tolerized mice. Both tolerized granulocytes and tolerized B cells significantly suppressed the production of anti-DNA *in vitro*. In granulocytes from tolerized mice compared to saline-treated littermate controls, real-time PCR analysis indicated that expression of *interferon-induced TNFAIP2* increased more than 2-fold while *Ptdss2* and *GATA1* mRNA were up-regulated more than 10-fold. In contrast, expression of these genes was significantly down-regulated in tolerized B cells. Further, another IFN-induced protein, Bcl2, was reduced in tolerized B cells as determined by Western blot analyses. In contrast, expression of FoxP3 was significantly increased in tolerized B cells. Together, these data suggest that B cells and granulocytes are altered toward suppressive functions by *in vivo* tolerization of BWF1 mice with pCons and it is possible these cell types participate in the clinical benefits seen *in vivo*.

Keywords: regulatory B cells, immune tolerance and regulation, pConsensus peptide (pCons), systemic lupus erythematosus, Anti-DNA Ab, polymorphonuclear cells (PMNs), granulocytes

OPEN ACCESS

Edited by:
Ciriaco A. Piccirillo, McGill University, Canada

Reviewed by:
Bhagirath Singh, Western University, Canada
Fan Pan, Chinese Academy of Sciences (CAS), China

****Correspondence:***
Ram P. Singh
ram.singh@va.gov; rpsingh11@hotmail.com

†Present address:
Ram P. Singh, Research Service,Veteran Administration Greater Los Angeles Healthcare System, Los Angeles, CA, United States

Specialty section:
This article was submitted to Immunological Tolerance and Regulation, a section of the journal Frontiers in Immunology

Received: *01 February 2021*
Accepted: *28 April 2021*
Published: *19 May 2021*

Citation:
Singh RP, Hahn BH and Bischoff DS (2021) Effects of Peptide-Induced Immune Tolerance on Murine Lupus. Front. Immunol. 12:662901. doi: 10.3389/fimmu.2021.662901

INTRODUCTION

Regulatory B cells and regulatory polymorphonuclear leukocytes (PMNs-granulocytes) influence immunity but are not well understood in systemic autoimmunity. Lupus causes significant morbidity, mortality, and economic loss. Systemic lupus erythematosus (SLE) is probably initiated by autoantibodies (*e.g.* anti-DNA) and immune complexes that induced inflammation and organ damage (1). One of the organs affected in some patients with SLE is the kidney and lupus nephritis is a leading cause of end stage kidney disease and death. Approximately two million people suffer from this disease, with the majority of cases being women of childbearing age. Lupus is a gender-biased disease with a female to male ratio of 9:1. African-American women are three times more likely to get lupus than Caucasian women. Lupus is also more common in Hispanic, Asian, and Native American women than in Caucasians. In the last 50 years, there have been only two new lupus-specific therapy approved by the FDA, Benlysta (anti-BAFF, and voclosporin (a calcineurin inhibitor). Current treatments, including the newer ones, rarely induce sustained disease remission. Therefore, additional treatment strategies are urgently needed. The modulation of abnormal immune regulation is an object of intense investigation in several experimental autoimmune diseases with the goal to limit the numbers of abnormal pathogenic cells and autoantibodies, and to achieve restoration of immune system self-tolerance by the administration of peptides that induce regulatory cells.

We have focused studies in a mouse model of SLE, the BWF1-New Zealand Black/New Zealand White (NZB/NZW) mouse, which has several characteristics in common with human SLE (2, 3). These mice spontaneously develop fatal immune mediated glomerulonephritis with high titers of anti-nuclear antibodies including high affinity IgG antibodies to dsDNA and show female to male bias. In this model, we used a peptide, pCons, to induce regulatory cells which are protective in SLE. We studied gene expression in splenocytes using Affymetrix microarray analysis (448 genes were differentially regulated one week after tolerance induction), followed by validation studies with quantitative real-time RT-PCR in $CD4^+$ and $CD8^+$ T cell subsets. In the current study, we test for potential molecular mechanisms that govern the function of tolerized B cells (including B_{regs}) and PMNs-granulocytes in this model.

Regulatory B cells (B_{regs}), a novel subpopulation of B cells, are a significant area of research due to their therapeutic relevance, immune regulatory function, and ability to sustain self-tolerance (4–6). Evidence suggesting a role for (B_{regs}) in the immune system has been described since the 1970s. These studies suggest that there is a potential role for B_{regs} in reducing T cell activity and inducing immune tolerance (7–10). Over the past decade, B_{regs} have been identified in many autoimmune diseases (11–17). Approaches to manipulate B cells in a manner that is beneficial in attenuating inflammatory and autoimmune conditions, including SLE, are not clear. The mechanisms by which B_{regs} influence the functions of $CD4^+$ and $CD8^+$ T_{regs} are not known. Additionally, genes expressed in B_{reg} cells (other than IL-10) that offer protective responses and their molecular mechanisms of function remain to be defined.

Polymorphonuclear neutrophils (PMN-granulocytes) have been shown to play a role in a variety of autoimmune diseases including Rheumatoid Arthritis (18), Inflammatory Bowel Disease (19, 20), and Experimental Autoimmune Encephalomyelitis (EAE) (21). Studies have shown that PMNs-granulocytes are capable of interacting with T cells by presenting class I and class II restricted antigens on their surface (22–26) as well as in a non-MHC restricted fashion (22). PMNs-granulocytes have also been shown to express the costimulatory molecules CD80 and CD86 (27), the regulation of which is important in autoimmunity and immune tolerance. In patients with SLE, granulocytes undergoing NETosis are increased, and the nets contain DNA/nucleosome/proteins that promote autoreactivity and production of type 1 IFNs (28). The exact mechanisms of PMNs-granulocytes and B_{regs} interaction with other regulatory cells and their cross-talk are currently poorly defined. In this report, we provide novel evidence that pCons tolerance induces $CD4^+FoxP3^+$ T cells and potent regulatory B cells and granulocytes capable of suppressing autoimmunity *in vitro* in a murine model of SLE. Understanding the role of regulatory T cells, B cells and granulocytes may provide novel mechanistic insight for SLE and expand our knowledge of immune tolerance and can identify potential new targets for SLE.

MATERIALS AND METHODS

Mice

NZB (H-2d/d), NZW (H-2z/z) and NZB/NZW F1 (H-2d/z) mice were purchased from the Jackson Laboratories (Bar Harbor, ME, USA) or bred at the University of California Los Angeles (UCLA). All mice were treated in accordance with the guidelines of the University of California Los Angeles Animal Research Committee, an Institution accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). Mice were housed in pathogen-free conditions. Female mice were used for all experiments.

Peptides

The peptides used in this study and the MHC molecules they bind have been described earlier (29, 30). pCons (FIEWNKLRFRQGLEW), the artificial tolerizing peptide, contains T-cell determinants based on the J558 VH regions of several murine anti-dsDNA Ab from BWF1 mice (29, 31–35). Peptides were synthesized at Chiron Biochemicals (San Diego, CA, USA), purified to a single peak on high-performance liquid chromatography, and analyzed by mass spectroscopy for expected amino acid content.

Treatment of Mice

Ten- to twelve-week-old BWF1 mice received a single i.v. dose of 1 mg of pCons, dissolved in saline, as reported previously (29, 31, 36) for tolerance induction. For immunophenotyping of regulatory B cells, female 35-wk-old BWF1 mice were used and injected with pCons (1 mg *i.v.*). After 3 days, blood was obtained, RBC lysed, and cells were stained with CD19, CD1d and CD5

antibodies and FACS performed. Control mice received either a similar amount of pNeg (negative control peptide) or saline.

Cell Isolation, Preparation, Immunophenotyping, and Flow cytometry

Spleen cells were isolated ~1 week after administration of the pCons peptide from tolerized, saline-treated, or naïve BWF1 mice. Single cell suspensions of splenocytes were prepared by passing cells through cell strainers (Fisher). ACK lysing buffer, (Sigma, St Louis, MO, USA) was used to lyse red blood cells. Cells were washed and re-suspended in RPMI complete media. Cell subsets were further enriched following incubation with anti-CD4 (L3T4), anti-B (CD45R/B220), anti-CD8 (CD8a Ly-2), anti-NK1.1 (CD95b), anti-DX5, anti-CD11C, and anti-Gr-1 microbeads from Miltenyi Biotech (Auburn, CA, USA). Purity of cells was determined to be more than 90% pure as assessed by flow cytometry (FACS). For immunophenotyping, isolated cells were washed with FACS buffer and 1–2 million cells were used for surface staining. Before staining, cells were incubated with rat anti-mouse CD16/CD32 (FC III/II receptor) Ab to block nonspecific binding.

For regulatory B cell immunophenotyping, female 35 wk-old BWF1 mice were treated with pCons 1 mg *i.v.* and blood was obtained after (3-5 days). Splenocytes were depleted of red blood cells (RBCs) and then stained with CD19, CD1d, and CD5 antibodies for FACS analysis. Antibodies for cell surface staining and isotype controls were from BD Biosciences, BD Pharmingen, eBiosciences, or BioLegend. CD4 (L3T4), CD25 (PC61.5) and CTLA-4 (UC10-4F10-11) staining was performed with antibodies from BD Pharmingen. FoxP3 (FJK-16s) staining was performed with an eBiosciences intracellular kit. Data were collected using FACSCalibur (BD Biosciences) and analyzed by BD Cell Quest software (Becton-Dickinson, Mountain View, CA) or FCS De Nova software (Thornhill, Ontario, Canada).

Western Blot Analysis

Western blot analyses were performed as described earlier (37). In brief, cell lysates were prepared from the naïve and tolerized B cells from the splenocytes of naïve and pCons-treated BWF1 mice. Cells were lysed with RIPA buffer (150 nM NaCl, 1.0% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 10 mM Tris, pH 7.3) supplemented with Protease Arrest protease inhibitor cocktail solution (G Biosciences, Maryland Heights, MO, USA). Protein was measured from each sample using the Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA) and an equal amount of protein was loaded in each well. The lysates were resolved on a 4–12% NuPage gel (Invitrogen, Carlsbad, CA, USA) in reducing conditions. Proteins were electro-transferred onto a polyvinylidene fluoride membrane (Invitrogen). The membranes were blocked with 3% BSA and immunoblotted with a specific antibody, bcl2 (50E3), (1:200 dilution; Cell Signaling Technology, Inc.) or β-actin (1:100 000 dilution; Sigma, Inc.). Following washing, the membranes were incubated in secondary antibodies (1:2500 dilution; Santa Cruz Inc, Santa Cruz, CA, USA). All blocking, incubation and washing steps were performed in TBST (TBS and 0.1% Tween 20). Proteins were visualized using ECL (GE Healthcare, Buckinghamshire, UK).

RNA Isolation and Real-Time PCR

Total cellular RNA was isolated from purified cell subsets or total splenocytes from saline-treated or pCons-tolerized BWF1 mice with TRIzol (Invitrogen, Carlsbad, CA, USA) as per manufacturer's protocols. Real-time PCR was performed as described earlier (29, 33–35). Each experimental group consists of the pooled spleen cells of 3–4 mice from each group. 100 ng of total RNA was used with one-step RT-PCR reagents (Applied Biosystems, Foster City, CA, USA). Quantitative real-time reverse transcription was performed using TaqMan technology on an ABI Prism 7900 HT Sequence Detection System (Applied Biosystems). Primers and probes of *IFI205*, *GATA1*, *Ptdss2*, *TNFip2*, *FoxP3* and *GAPDH* were obtained from Applied Biosystems, Foster City, CA, USA. The oligonucleotide sequences used for the primers and TaqMan probes (Applied Biosystem, Foster City, CA) are described (29, 33–35). GAPDH was used as an endogenous control in each experimental set.

Cell Culture and Measurement of Anti-DNA Antibodies

Assays were performed to measure anti-DNA Ab as described earlier (29, 31, 34, 35, 37). For optimal Ab production, B cells ($1x10^5$ cells) from keep old (40-50-wk-old) naïve BWF1 females with 3+ proteinuria or higher, $CD4^+CD25^-$ T cells ($1x10^6$) from young 10–12-wk-old naive BWF1 females without proteinuria, naïve $CD8^+$ T cells ($1x10^6$), and irradiated APC ($1x10^5$) cells were isolated and cultured with granulocytes or B cells ($1x10^6$) from tolerized mice or controls. Cell cultures were performed in RPMI 1640 supplemented with L-glutamine (2 mM), penicillin (100 units/ml), streptomycin (0.1 mg/ml), 2-mercaptoethanol (Gibco) and 10% fetal bovine serum (FBS). For tolerized B cells analysis, we cultured, as indicated above pCons-tolerized B cells ($1x\ 10^6$) with naïve $CD4^+CD25^-$ T cells ($1x\ 10^6$), naïve B ($1x\ 10^5$) and/or naïve $CD8^+$ T cells ($1x10^6$) cells. After 72-96 hours, culture supernatants were obtained and anti-DNA IgG was measured by ELISA.

Statistical Analyses

Data were analyzed using Prism 4.0 (GraphPad Software, San Diego, CA). Comparisons were performed using paired one- or two-tailed test. Nonparametric testing among more than two groups was performed by one-way ANOVA. Results are expressed as mean ± SEM. $p<0.05$ was considered significant.

RESULTS

pCons-Induced Tolerized B Cells and Granulocytes Suppressed Anti-DNA Ab Production by BWF1 Cells

To our knowledge, no studies have been performed to address the role of regulatory B cells and granulocytes in the immune tolerance and BWF1 lupus. To address this, we harvested B cells and granulocytes from the spleens of naïve and tolerized BWF1 mice 7 days after the induction of tolerance (peptide treatment 1 mg *i.v.* once a week). We used *in vitro* assays to test the effects of each cell type on anti-DNA Ab production with the addition of naïve $CD4^+$ helper cells ($CD4^+CD25^-$ T cells) plus naïve B cells

(from old BWF1 nephritic mice) and naïve $CD8^+$ T cells to each cultured subset either separately or in combination with tolerized B cells (B_{regs}) and granulocytes. Anti-DNA Ab was analyzed as described previously (29, 31, 34, 35, 37). Briefly, purified populations of different spleen effector cell subsets including B cells, naïve $CD4^+$T cells ($CD4^+CD25^-$Tcells), naïve $CD8^+$ T cells, and tolerized granulocytes and B cells were harvested (using the appropriate Miltenyi Biotec microbeads via AutoMACS) one week after pCons treatment of BWF1 mice. Naïve $CD4^+$ T cells from young mice (10-12-weeks-old) and B cells from old nephritic BWF1 mice (40-50-weeks-old with 3+ proteinuria) were co-cultured in complete medium with tolerized/regulatory B cells and granulocytes and other effector cell types such as (naïve $CD8^+$ T cells) from spleens of tolerized mice. Our previous cell-dose response study with $CD8^+$ Treg showed that 1×10^6 cells are optimum in mixed cell culture experiments (29) therefore, we used the same number of cells in these experiments. In addition previously, we had found that a ratio of B cells to helper T cells ($CD4^+CD25^-$T cells) to regulatory/suppressor T cells of 1:10:10 are needed to observe optimal suppression of anti-DNA antibodies (29, 32, 35). Therefore, we used this same ratio with each effector cell type. We found that both tolerized B cells and granulocytes suppressed the production of anti-DNA Ab (**Figures 1** and **2**). Although we did not determine whether this suppressive effect was direct or indirect on autoreactive B cells through $CD4^+$ or $CD8^+$ T cells or by synergistic effect by those cells with tolerized B cells and granulocytes, this data clearly suggests that pCons-induced regulatory B cells and granulocytes suppress the anti-DNA Ab production and thus play a significant role in autoimmunity.

Microarray Analysis Showed Altered Regulation of Genes in Non-T Cells

Since our previous published microarray data showed that pCons treatment induces major changes in white blood cells (WBC) subsets of BWF1 spleen cells [448 genes differentially-regulated in whole splenocytes of tolerized compared to control mice (33)], we were interested to see the potential role of regulatory B cells and granulocytes. Thus, in this report, we characterized expression of selected genes (highly upregulated) in different cell populations in non-T cells (tolerized B cells and tolerized granulocytes cells) and further tested the ability of these cell subsets to suppress production of anti-DNA Ab in lupus. Our data suggests that cell types other than T cells may play major roles in this model of immune tolerance.

B cells and Granulocytes Produced Significantly Increased/Decreased Amounts of mRNA for Several Genes of Interest Including Interferon Genes After pCons Treatment in BWF1 Lupus Mice

To address the role of tolerized non-T cell subsets after pCons treatment in BWF1 mice, B cells and granulocytes were obtained from the spleen of BWF1 mice 1 weeks after pCons treatment. RNA was isolated from these cell subsets and real-time PCR was performed as described earlier (33). Real-time PCR analyses

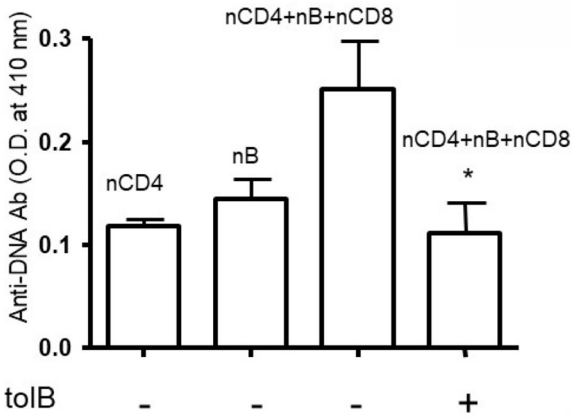


FIGURE 1 | Anti-DNA Ab was significantly decreased in the presence of tolerized B cells. Naïve $CD4^+$ T cells, CD8+ T cells and $CD45R/B220^+$B cells were isolated from BWF1 mice spleen cells using microbeads from Miltenyi Biotech (Auburn, CA, USA). Cells were cultured in RPMI 1640 supplemented with L-glutamine (2 mM), penicillin (100 units/ml), streptomycin (0.1 mg/ml), 2-mercaptoethanol (Gibco) and 10% fetal bovine serum (FBS). Cells were co-cultured in the presence of tolerized B cells ($1x10^6$ cells). Different immune cell subsets (naïve B cells, $1x10^5$ cells from old nephritic mice; $CD4^+CD25^-$ T cells, $1x10^6$; naïve $CD8^+$ T cells, $1x10^5$) were isolated from splenocytes and cultured with tolerized B cells ($1x10^6$ cells). After the 72-96 hours range, culture supernatants were obtained. Anti-DNA Ab levels were measured from culture supernatants by ELISA. *$p < 0.05$.

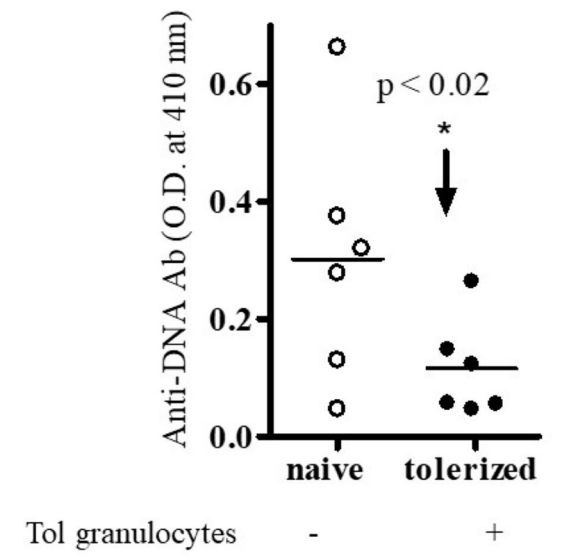


FIGURE 2 | Anti-DNA Ab was significantly decreased in the presence of tolerized PMNs-granulocytes. Different immune cell subsets (naïve B cells, $1x\ 10^5$ cells from old nephritic mice; $CD4^+CD25^-$ T cells, $1x10^6$; naïve $CD8^+$ T cells, $1x10^5$) were isolated and cultured with tolerized granulocytes (GR, $1x10^6$). Cell subsets were isolated from total spleen cells of BWF1 mice. Cells were cultured in RPMI 1640 supplemented with L-glutamine (2 mM), penicillin (100 units/ml), streptomycin (0.1 mg/ml), 2-mercaptoethanol (Gibco) and 10% fetal bovine serum (FBS). After the 72- 96 hours range, culture supernatants were obtained. Anti-DNA Ab levels were measured from culture supernatants by ELISA. *$p < 0.05$.

indicate that *TNFAIP2 (*Tumor necrosis factor alpha-induced protein 2) was increased ~2-fold (**Figure 3A**), and *Ptdss2 (*Phosphatidylserine synthase 2 *and GATA1 (*GATA-binding factor 1) *mRNA were upregulated more than 10-fold in tolerized granulocytes compared to naïve granulocytes* (**Figures 3B, C**). In contrast, the mRNA of all the above genes (**Figures 3D–F**) and those of IFN-induced genes including *IFI203* and *IFI205* (**Figures 3G, H**) were down regulated in tolerized B cells. Although, the decreased level of *IFI203 and IFI205* did not reach to the significance. However, other IFNs genes were significantly decreased. Thus, this data displays dynamic interplay and suggests that pCons has differential effects on different interferon genes in our tolerance model. Collectively these data demonstrate that pCons treatment modify the interferon's gene signature differentially in tolerized B cells and granulocytes.

pCons-Tolerized B Cells From Lupus Mice Have Increased FoxP3 mRNA and Bcl2 Protein Levels

The transcription factor forkhead box P3 (FoxP3), also known as scurfin, plays an important role in the maintenance of immunological homeostasis and restoration of self-tolerance. Dysfunction and mutations of the *FoxP3* gene causes immunodysregulation polyendocrinopathy enteropathy X-linked (or IPEX) syndrome. *FoxP3* also participates in maintaining the immune system response (38) and in the development and function of regulatory T cells (39–42). In the present study, we evaluated the expression of *FoxP3* in pCons-tolerized B cells of lupus (BWF1 mice) compared with naïve B cells. Surprisingly, we found significantly increased expression of *FoxP3* in tolerized B cells compared to naïve B cells (**Figure 3I**). Next, we investigated the protein expression of *bcl2* from cell lysates of tolerized B cells and naïve B cells with Western blot assay. We found that tolerized B cells have decreased levels of Bcl2 protein compared to naïve B cells (**Figure 3J**). Bcl-2 regulates cell death (apoptosis) by promoting or inhibiting apoptosis (43, 44). We have shown previously that $CD4^+$ and $CD8^+$ T cells from tolerized mice have significantly reduced apoptosis. Thus our data suggest that pCons tolerance may also affect apoptosis of B cells in our tolerance model and may play a significant role in survival of these cells by regulating immune tolerance.

pCons Treatment Induced and Modified the Cell Surface Expression Markers for Regulatory B Cells

Our previous study showed that pCons treatment induces both $CD4^+$ and $CD8^+$ regulatory T cells (29, 32, 36). Based on these data and our gene expression study (33), we hypothesize that pCons treatment may induce suppressor/regulatory B cells (B_{regs}) and granulocyte cells *with* the potential to suppress the proliferation of naïve $CD4^+CD25^-$ cells and naïve B cells as well as the production of anti-DNA Ab. To address this, we isolated spleen cells from female BWF1 mice after one week of pCons treatment (1 mg *i.v*) and performed immunophenotyping studies with flow cytometry from naïve and pCons-treated mice (**Figures 4A, F** live gating scheme). We found that pCons treatment of BWF1 mice increases percent expression of $CD19^+CD1d^+$ regulatory B cells including median fluorescence intensity (**Figures 4B, G, D, E**). This is an important finding because two previous studies have revealed similar phenotype of B_{regs} in SLE patients (45, 46). Further, we found that pCons treatment reduces the percent expression of $CD19^+CD5^+$ B cells (**Figures 4C, H, I**). The median fluorescence intensity (MFI) of $CD19^+$ $CD5^+$ cells were significantly decreased in pCons treated mice (**Figure 4J**). These data show that pCons treatment modified the B cells expression markers CD1d and CD5, and since we have also shown that $CD4^+$ and $CD8^+$ T cells from tolerized mice suppress autoreactive B cells and could account for their reduced numbers, this suggests that pCons treatment induces regulatory B cells.

pCons Treatment Increased $CD4^+FoxP3^+$ Regulatory T Cells and Significantly Reduced Percent Expression and Median Fluorescence Intensity of CTLA-4 (Cytotoxic T-Lymphocyte-Associated Proten-4) in $CD8^+$ T Cells of BWF1 Lupus Mice

We were interested to see whether pCons treatment induces regulatory T cells and whether it affects CTLA-4 expression. CTLA-4 plays an important role in immune tolerance and T-cell activation. We found that pCons treatment significantly increased the number of $CD4^+FoxP3^+$ T cells in BWF1 mice compared to naïve and/or saline-treated mice (**Figures 5A–D, F–H**). We also measured the CTLA-4 expression on T cells ($CD8^+$T cells) and found that percent expression of CTLA-4 was significantly decreased in pCons treated mice (**Figure 5E**) Further, we found that pCons treatment significantly reduced the median fluorescence intensity (MFI) of CTLA-4 expression in $CD8^+$ T cells compared to naïve mice (**Figures 5I, J**). Thus, the data shows the immunomodulatory role of pCons in BWF1 mice. However, future study is warranted to pinpoint the exact mechanism of pCons activity in Lupus.

DISCUSSION

In the present study, we have added to previous work showing that pCons induces $CD8^+$ and $CD4^+$ suppressive cells and shown that B cells and granulocytes from tolerized mice suppress anti-DNA Ab production *in vitro*. Several suppressive mechanisms/ factors may be involved including IL-10, TGFβ, IL-35, and combinations of TLR9, CD40, and/or B cell receptor (BCR) and engagement of CD80/CD86 on B_{regs} (45, 47). pCons treatment significantly increased the number of $CD4^+FoxP3^+$ T cells. In earlier studies, we showed that these $FoxP3^+$ T cells (both $CD4^+$ and $CD8^+$ T_{reg}) suppressed autoimmunity *in vivo* and anti-DNA production *in vitro* (29, 32, 36). Immune tolerance induced by pCons prolonged survival of BWF1 lupus mice (NZB/NZW) F1 and delayed the appearance of glomerulonephritis (29, 31, 35). The pCons-induced regulatory T cells suppressed proliferation of naïve $CD4^+$ T cells and naïve $CD19^+/B220^+$ B cells and the production of anti-DNA antibodies (29, 32, 34–36).

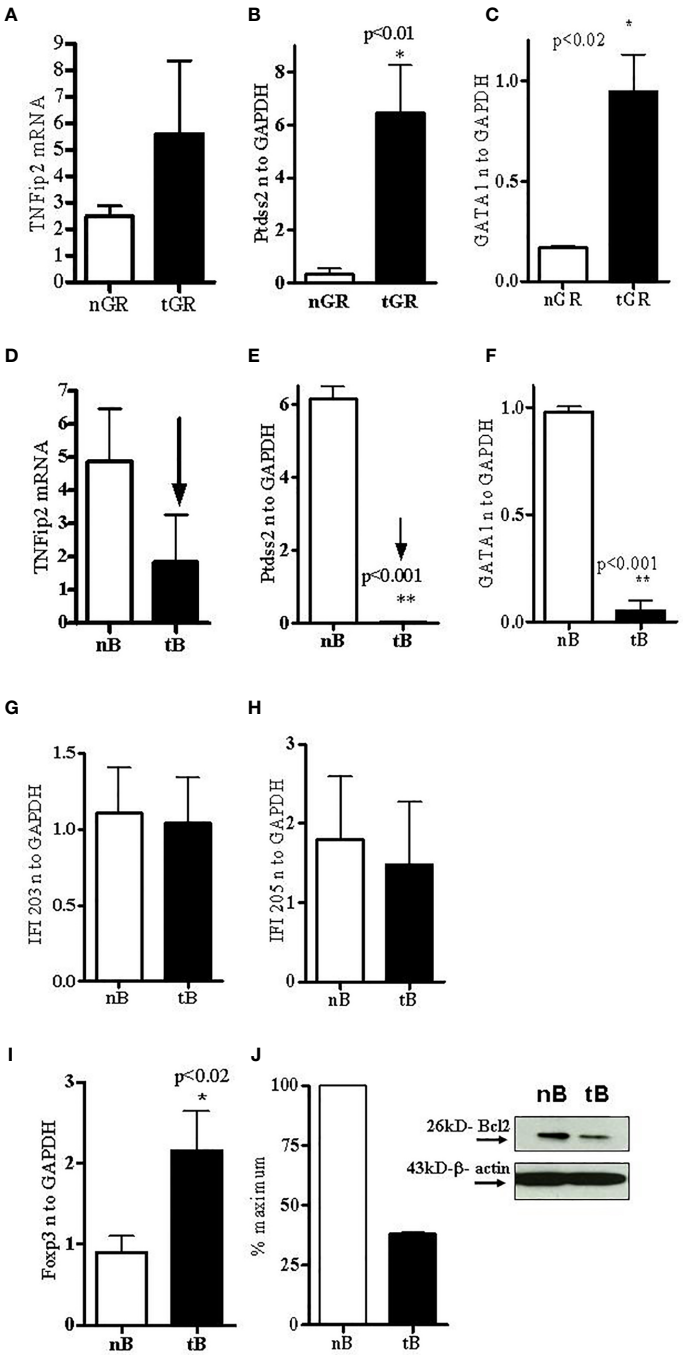


FIGURE 3 | Tolerized B cells have reduced IFNs gene mRNA and Bcl2 protein level and increased FoxP3 mRNA expression. RNA was isolated from naïve and tolerized B cells and granulocytes. Real time PCR was performed with 100 ng of RNA with gene specific primers and probes. Data was normalized with GAPDH mRNA levels. *p < 0.05. *TNFAIP2*, *Ptdss2*, and *GATA1* mRNA was increased **(A–C)** in tolerized granulocytes (GR) but reduced in tolerized B cells **(D–F)**. *IFI203* and *IFI205* was decreased in tolerized B cells **(G, H)**. **(I)** *FoxP3* expression was increased in tolerized B cells. **(J)** Quantification of Western blot analysis of Bcl2 protein levels in cell lysates from naïve and tolerized sorted B (CD45R/B220) cells.

T cell suppressive capacity correlated with modulation of Mitogen-Activated Protein kinase (p38 MAP kinase) activity and FoxP3 expression in $CD4^+$ Tregs (48). In the current study we found that CTLA-4 median fluorescence intensity was significantly decreased in the $CD8^+$ T cells of pCons-treated BWF1 mice. This is a significant finding as CTLA-4 is involved in negative signaling and plays a pivotal inhibitory role in T cell anergy and prevention of autoimmunity. In addition, recent

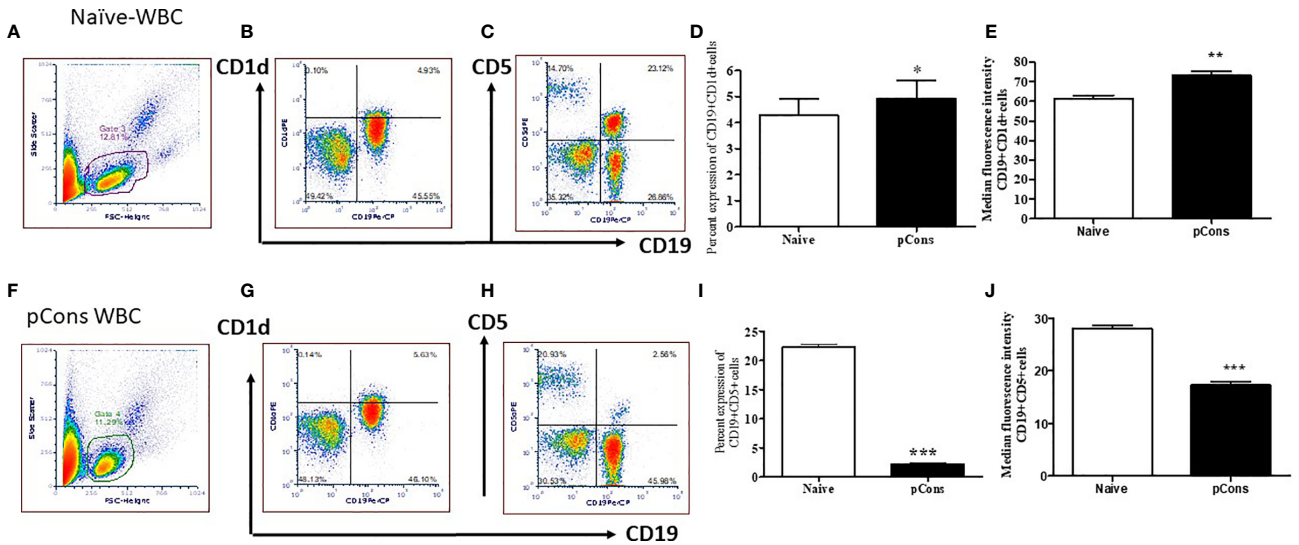


FIGURE 4 | pCons treatment modified the cell surface expression markers for regulatory B cells. Female 35-wk old BWF1 mice were treated with pCons (1 mg i.v.). After 3 days, blood was obtained, RBC lysed, and cells were stained with CD19, CD1d and CD5 antibodies and FACS performed. Representative live cell gating strategy **(A, F)** and FACS analysis of CD1d **(B, G)** and CD5 **(C, H)** expression levels from representative naïve **(A–C)** and pCons treated **(F–H)** mice. Percent expression of $CD19^+$ $CD1d^+$ cells in naïve vs pCons treated mice **(D)**. Percent expression of $CD19^+CD5^+$cells in naïve vs pCons treated mice **(I)**. Quantification of Median Fluorescent Intensity of naïve and pCons-treated mice for CD1d **(E)** (from B and G Gate 3 and 4) and CD5 **(J)** (from C and H Gate 3 and 4). CD1d increased and CD5 cells decreased with pCons treatment. *p < 0.05, **p < 0.001, ***p < 0.0001.

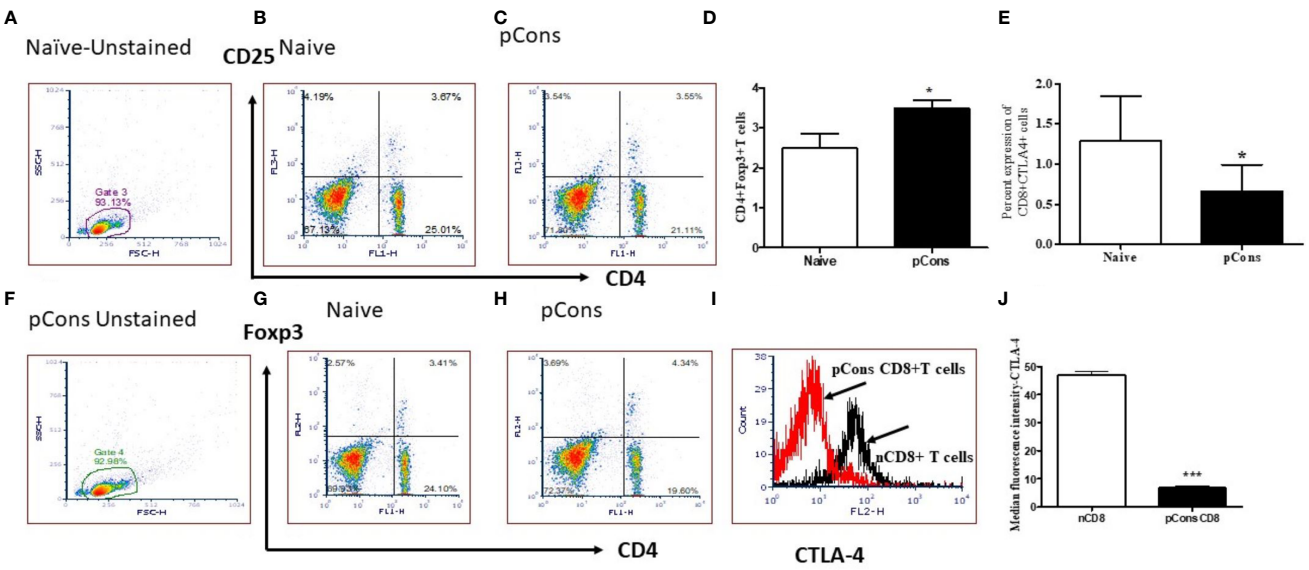


FIGURE 5 | pCons treatment increased $CD4^+FoxP3^+$ regulatory T cells and significantly reduced Median Fluorescence Intensity of CTLA-4 (Cytotoxic T-lymphocyte-Associated Proten-4) in $CD8^+$ T cells of BWF1 lupus mice. Female 12-20 wk-old BWF1 mice were treated with pCons (1mg i.v.). After 1-2 weeks, splenocytes were obtained, RBC lysed, and cells were stained with CD4, CD8, CD25, CTLA-4 and FoxP3 antibodies and FACS performed. 10,000 minimum cells were gated. Representative gating strategy **(A, F)** and FACS analyses of $CD4^+CD25^+$ **(B, C)**, $CD4^+FoxP3^+$ **(G, H)** and cumulative two-three experiments data for $CD4^+FoxP3^+$ cells **(D)** are shown. Cumulative data of $CD8^+$ $CTLA\text{-}4^+$ T cells experiments (two-three) is shown **(E)**. CTLA-4 staining (MFI) on $CD8^+$ T cells is shown **(I, J)**. $CD4^+FoxP3^+$T cells are significantly increased. CTLA-4 MFI is significantly decreased. *p < 0.05, **p < 0.001, ***p < 0.0001.

studies show that CTLA-4 controls follicular helper T cells and regulatory T cells, thereby controlling the B cells responses and humoral immunity (49–51). CTLA-4 also downregulates CD80 and CD86 on antigen presenting cells (APC); thus, altering the level of CD28 engagement on follicular helper T cells (51). However, its precise mechanism of action has not been fully

resolved. Recently abnormal CTLA-4 gene polymorphisms and function has been reported in SLE patients (52, 53).

For the first time to our knowledge, in this system we found that B cells and granulocytes also can be "tolerized" and subsequently function as regulatory/suppressor cells to prevent production of autoantibodies. In the current experiments, we used whole tolerized B cells and granulocytes for the suppression assay. We acknowledge the next step is to test the specific B_{regs} subsets to determine cell specificity and their mode of action and mechanism. Therefore, detailed molecular and cellular mechanisms of regulatory B cells and granulocytes are not completely clear and future study will be required to address this shortcoming.

Regulatory B cells and regulatory granulocytes are not well characterized in this SLE tolerance model and this study provided first novel mechanistic insight. We showed that 1) pCons tolerance altered expression of several candidate genes (see below) including interferon genes in tolerized B cells and granulocytes compared to naïve cells; 2) pCons tolerance modified the cell surface expression of regulatory B cells (and/or deleted the $CD19^+CD5^+$ subset); 3) pCons tolerance increased the percent expression of ($CD19^+CD1d^+$) cells; and 4) pCons-tolerized B cells and granulocytes significantly reduced the production of anti-DNA antibody in cell culture experiments of lupus mouse cells.

pCons tolerance has been shown to affect various genes and markers, cell surface molecules, cytokines and different cell types including regulatory T cells (both CD4 and CD8) (29–35, 37). In the present study, we showed that pCons induced B cells enriched in markers identifying suppressor B cells and these cells have significantly reduced interferon-induced genes (*IFN*) such as, *ptdss2*, *GATA1*, and T*NFip2* (**Figure 3**) compared to naïve mice. In contrast, we also found that these genes were significantly increased in tolerized granulocytes, with the exception of *TNFip2* which was upregulated but did not reach the significance level (**Figure 3**) demonstrating differential effect of pCons. Thus, our data indicate dynamic interplay of these genes or their gene products in different immune cell subsets in our pCons-induced tolerance model. How this interplay affects the overall immune response in lupus mice is not clear. However, recent studies have shown the importance of interferon genes in lupus (54–58). Lupus is characterized by the dysregulation of both the innate and the adaptive immune systems. An increased expression of type I IFN-regulated genes, termed IFN signature, has been reported in the majority of patients with SLE (59–61). In agreement with our findings, another study found that a tolerogenic peptide of the light chain complementarity-determining region 1 (hCDR1) down-regulates the expression of interferon-alpha (IFN-α) in murine and human SLE (62). IFN-α plays a major role in SLE pathogenesis and the levels of IFN-α were increased and correlated with SLE disease activity in the sera of mice and humans (63–65). Administration of exogenous IFN-α leads to worsening of disease in various mouse models (66). Type 1 IFN contributes to loss of tolerance and increases production of autoantibodies (67), induces differentiation of monocytes to myeloid-derived dendritic cells (mDC) (56), and plays a vital role in the activation of autoreactive T and B cells (68). Activation of TLR7 and TLR9 is thought to be central to induction of the type 1 IFN response (69, 70). Indeed, a recent therapeutic option in patients with lupus is through inhibition of type IFN–α and several recent clinical trial data suggest therapeutic benefit (71–73). Thus, our findings that pCons tolerance reduces the IFN genes in our lupus model has direct clinical and translational significance.

We have shown earlier that pCons peptide delayed the onset of autoimmunity in lupus mouse model by inducing immune tolerance and up-regulating FoxP3 in T cells which are suppressive (31, 34, 74). Other studies have reported that peptides from CDRs of pathogenic anti-DNA Ab could also prevent autoantibody production and down-regulate autoreactive T cell responses (75, 76). Similar to our results, these studies showed that a peptide derived from the CDR1 of a human anti-DNA Ab (hCDR1) could ameliorate lupus by inducing T_{regs} and suppressing the activation of autoreactive T cells through mechanisms including downregulation of transcription factors responsible for negative regulation of T-cell activation in lupus animal models (62, 76–79). Furthermore, clinical trial data has indicated safety and efficacy of hCDR1 (edratide) in SLE patients (80).

We demonstrated with flow cytometry (FACS) immunophenotyping that after pCons treatment the $CD19^+CD1d^+$ regulatory B cell subset was significantly increased in BWF1 lupus mice compared to naïve mice (**Figures 4B, G, D**). However, the $CD19^+CD5^+$ B cell subset was significantly decreased (**Figures 4C, H, I**). This is a significant finding and the next step will be to decipher the mechanisms with future functional studies including testing B cells from anti-CD5 treated control mice. In agreement with our experiments, a previous study showed that anti-CD5 therapy decreases severity of established disease in collagen-induced arthritis in DBA/1 mice (81). Thus, our data with pCons therapy has clinical and therapeutic relevance in peptide induced immune tolerance. We also found that tolerized B cells have significant increased *FoxP3* mRNA. Another study reported that the expansion of $CD25^{+hi}CD5^+$ and $FoxP3^+$ regulatory B cells is associated with SLE disease activity in humans (82). Similarly, the presence of $FoxP3^+$ $CD19^+CD5^+$ B cells in human peripheral blood mononuclear cells has also been reported (83). The diverse suppressive mechanisms of these regulatory B cells are through IL-10, TGFβ, and IL-35. Previously we have demonstrated that pCons-induced splenocytes have significantly increased amount of TGFβ, smad2, and smad3 expression and tolerized total $CD8^+$ T cells have increased amount of IL-10 (37). Although, we did not measure the expression of IL-10, TGFβ and IL-35 in the regulatory B cells in our model, it is tempting to speculate that these molecules will play important role in our system based on our previous data. Thus, our findings may suggest that pCons tolerance promotes tolerized B cells that can suppress the autoimmune responses. Similar to our study, hCDR1 tolerance has effects on B cell activating factor (BAFF) and B-cell CD74 macrophage inhibitory factor in murine lupus (84, 85). The reduced levels of BAFF correlated with reduced rate of maturation and differentiation of B cells and decrease in integrin expression. Recent studies provided further evidence of targeting of BAFF/BLys and APRIL in the management of lupus (86–88); and another study reported the effect of hCDR1 on IL-7 and apoptosis (89) and showed the rate of

apoptosis is reduced with hCDR1 treatment in lupus mice. Bcl2 and Bcl-$_{XL}$ levels were further reduced, and this was associated with reduced activation of T and B cells (90). We demonstrated earlier that pCons-induced CD8$^+$ T$_{regs}$ are resistant to apoptosis (29, 34). In the present study, we found that bcl2 protein level was significantly decreased in tolerized B cells compared to naïve B cells thus affecting the survival of these cells in BWF1 mice. This is in agreement with another study that revealed increased expression of Bcl2 leads to development of SLE like symptoms in Bcl2 transgenic mice (91). Thus, altogether, our data suggests that pCons' effect on tolerized B cells and down-regulation of IFNs and bcl2 may play overall therapeutic beneficial effects in our tolerance model.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by UCLA Animal Research Committee (ARC).

AUTHOR CONTRIBUTIONS

RPS contributed to the experimental design, obtaining funding, conducting experiments, analyzing data, preparing figures, and writing of the manuscript. BHH contributed to funding and editing of the manuscript. DSB contributed to figure and manuscript editing. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the NIH grants AR54034, AI 083894, AI65645 to RPS, UCLA Senate Core Grant to BHH and RPS, and UCLA Oppenheimer Clinical Seed Grant and American Autoimmune Related Disease Association grant to RPS.

ACKNOWLEDGMENTS

We thank the UCLA Flow Cytometry Core Facility for cell sorting and FACS experiments, Dr. Desmond Smith's Lab for the real time PCR, Dr. Ravi Dinesh for technical help, and Dr. Antonio La Cava for intellectual insights.

REFERENCES

1. Kotzin BL. Systemic Lupus Erythematosus. *Cell* (1996) 85:303–6. doi: 10.1016/S0092-8674(00)81108-3
2. Wakeland EK, Liu K, Graham RR, Behrens TW. Delineating the Genetic Basis of Systemic Lupus Erythematosus. *Immunity* (2001) 15:397–408. doi: 10.1016/S1074-7613(01)00201-1
3. Theofilopoulos AN, Dixon FJ. Murine Models of Systemic Lupus Erythematosus. *Adv Immunol* (1985) 37:269–390. doi: 10.1016/S0065-2776(08)60342-9
4. Karim MR, Wang YF. Phenotypic Identification of CD19(+)CD5(+)CD1d(+) Regulatory B Cells That Produce Interleukin 10 and Transforming Growth Factor Beta1 in Human Peripheral Blood. *Arch Med Sci* (2019) 15:1176–83. doi: 10.5114/aoms.2018.77772
5. Li W, Tian X, Lu X, Peng Q, Shu X, Yang H, et al. Significant Decrease in Peripheral Regulatory B Cells is an Immunopathogenic Feature of Dermatomyositis. *Sci Rep* (2016) 6:27479. doi: 10.1038/srep27479
6. Mauri C, Menon M. Human Regulatory B Cells in Health and Disease: Therapeutic Potential. *J Clin Invest* (2017) 127:772–9. doi: 10.1172/JCI85113
7. Morris A, Moller G. Regulation of Cellular Antibody Synthesis Effect of Adoptively Transferred Antibody-Producing Spleen Cells on Cellular Antibody Synthesis. *J Immunol* (1968) 101:439–45.
8. L'Age-Stehr J, Teichmann H, Gershon RK, Cantor H. Stimulation of Regulatory T Cell Circuits by Immunoglobulin-Dependent Structures on Activated B Cells. *Eur J Immunol* (1980) 10:21–6. doi: 10.1002/eji.1830100105
9. Kennedy MW, Thomas DB. A Regulatory Role for the Memory B Cell as Suppressor-Inducer of Feedback Control. *J Exp Med* (1983) 157:547–58. doi: 10.1084/jem.157.2.547
10. Evans DE, Munks MW, Purkerson JM, Parker DC. Resting B Lymphocytes as APC for Naive T Lymphocytes: Dependence on CD40 Ligand/CD40. *J Immunol* (2000) 164:688–97. doi: 10.4049/jimmunol.164.2.688
11. Bouaziz JD, Yanaba K, Tedder TF. Regulatory B Cells as Inhibitors of Immune Responses and Inflammation. *Immunol Rev* (2008) 224:201–14. doi: 10.1111/j.1600-065X.2008.00661.x
12. Lund FE. Cytokine-Producing B Lymphocytes-Key Regulators of Immunity. *Curr Opin Immunol* (2008) 20:332–8. doi: 10.1016/j.coi.2008.03.003
13. Mauri C, Ehrenstein MR. The 'Short' History of Regulatory B Cells. *Trends Immunol* (2008) 29:34–40. doi: 10.1016/j.it.2007.10.004
14. Mizoguchi A, Bhan AK. A Case for Regulatory B Cells. *J Immunol* (2006) 176:705–10. doi: 10.4049/jimmunol.176.2.705
15. Serra P, Santamaria P. To 'B' Regulated: B Cells as Members of the Regulatory Workforce. *Trends Immunol* (2006) 27:7–10. doi: 10.1016/j.it.2005.11.003
16. Yanaba K, Bouaziz JD, Haas KM, Poe JC, Fujimoto M, Tedder TF. A Regulatory B Cell Subset With a Unique CD1dhiCD5+ Phenotype Controls T Cell-Dependent Inflammatory Responses. *Immunity* (2008) 28:639–50. doi: 10.1016/j.immuni.2008.03.017
17. Yanaba K, Bouaziz JD, Matsushita T, Tsubata T, Tedder TF. The Development and Function of Regulatory B Cells Expressing IL-10 (B10 Cells) Requires Antigen Receptor Diversity and TLR Signals. *J Immunol* (2009) 182:7459–72. doi: 10.4049/jimmunol.0900270
18. Iking-Konert C, Ostendorf B, Sander O, Jost M, Wagner C, Joosten L, et al. Transdifferentiation of Polymorphonuclear Neutrophils to Dendritic-Like Cells at the Site of Inflammation in Rheumatoid Arthritis: Evidence for Activation by T Cells. *Ann Rheum Dis* (2005) 64:1436–42. doi: 10.1136/ard.2004.034132
19. Premchand P, Takeuchi K, Bjarnason I. Granulocyte, Macrophage, Monocyte Apheresis for Refractory Ulcerative Proctitis. *Eur J Gastroenterol Hepatol* (2004) 16:943–5. doi: 10.1097/00042737-200409000-00023
20. Suzuki Y, Yoshimura N, Saniabadi AR, Saito Y. Selective Granulocyte and Monocyte Adsorptive Apheresis as a First-Line Treatment for Steroid Naive Patients With Active Ulcerative Colitis: A Prospective Uncontrolled Study. *Dig Dis Sci* (2004) 49:565–71. doi: 10.1023/B:DDAS.0000026299.43792.ae
21. Zehntner SP, Brickman C, Bourbonniere L, Remington L, Caruso M, Owens T. Neutrophils That Infiltrate the Central Nervous System Regulate T Cell Responses. *J Immunol* (2005) 174:5124–31. doi: 10.4049/jimmunol.174.8.5124
22. Prior C, Townsend PJ, Hughes DA, Haslam PL. Induction of Lymphocyte Proliferation by Antigen-Pulsed Human Neutrophils. *Clin Exp Immunol* (1992) 87:485–92. doi: 10.1111/j.1365-2249.1992.tb03024.x
23. Potter NS, Harding CV. Neutrophils Process Exogenous Bacteria Via an Alternate Class I MHC Processing Pathway for Presentation of Peptides to T Lymphocytes. *J Immunol* (2001) 167:2538–46. doi: 10.4049/jimmunol.167.5.2538
24. Tvinnereim AR, Hamilton SE, Harty JT. Neutrophil Involvement in Cross-Priming CD8+ T Cell Responses to Bacterial Antigens. *J Immunol* (2004) 173:1994–2002. doi: 10.4049/jimmunol.173.3.1994

25. Beauvillain C, Delneste Y, Scotet M, Peres A, Gascan H, Guermonprez P, et al. Neutrophils Efficiently Cross-Prime Naive T Cells In Vivo. *Blood* (2007) 110:2965–73. doi: 10.1182/blood-2006-12-063826
26. Culshaw S, Millington OR, Brewer JM, McInnes IB. Murine Neutrophils Present Class II Restricted Antigen. *Immunol Lett* (2008) 118:49–54. doi: 10.1016/j.imlet.2008.02.008
27. Muller I, Munder M, Kropf P, Hansch GM. Polymorphonuclear Neutrophils and T Lymphocytes: Strange Bedfellows or Brothers in Arms? *Trends Immunol* (2009) 30:522–30. doi: 10.1016/j.it.2009.07.007
28. Gupta S, Kaplan MJ. The Role of Neutrophils and NETosis in Autoimmune and Renal Diseases. *Nat Rev Nephrol* (2016) 12:402–13. doi: 10.1038/nrneph.2016.71
29. Hahn BH, Singh RP, La Cava A, Ebling FM. Tolerogenic Treatment of Lupus Mice With Consensus Peptide Induces FoxP3-expressing, Apoptosis-Resistant, TGFbeta-secreting Cd8+ T Cell Suppressors. *J Immunol* (2005) 175:7728–37. doi: 10.4049/jimmunol.175.11.7728
30. Hahn BHA, La Cava A. The Anti-DNA Ig Consensus Peptide Pcons Facilitates Regulatory T Cell Activity in SLE Patients. *Arthritis Rheum* (2007) 56:S546–547.
31. Hahn BH, Singh RR, Wong WK, Tsao BP, Bulpitt K, Ebling FM. Treatment With a Consensus Peptide Based on Amino Acid Sequences in Autoantibodies Prevents T Cell Activation by Autoantigens and Delays Disease Onset in Murine Lupus. *Arthritis Rheum* (2001) 44:432–41. doi: 10.1002/1529-0131(200102)44:2<432::AID-ANR62>3.0.CO;2-S
32. La Cava A, Ebling FM, Hahn BH. Ig-Reactive CD4+CD25+ T Cells From Tolerized (New Zealand Black X New Zealand White)F1 Mice Suppress In Vitro Production of Antibodies to DNA. *J Immunol* (2004) 173:3542–8. doi: 10.4049/jimmunol.173.5.3542
33. Singh RP, Dinesh R, Elashoff D, de Vos S, Rooney RJ, Patel D, et al. Distinct Gene Signature Revealed in White Blood Cells, CD4(+) and CD8(+) T Cells in (Nzbx NZW) F1 Lupus Mice After Tolerization With Anti-DNA Ig Peptide. *Genes Immun* (2010) 11:294–309. doi: 10.1038/gene.2010.6
34. Singh RP, La Cava A, Hahn BH. Pconsensus Peptide Induces Tolerogenic Cd8+ T Cells in Lupus-Prone (NZB X NZW)F1 Mice by Differentially Regulating FoxP3 and PD1 Molecules. *J Immunol* (2008) 180:2069–80. doi: 10.4049/jimmunol.180.4.2069
35. Singh RP, La Cava A, Wong M, Ebling F, Hahn BH. Cd8+ T Cell-Mediated Suppression of Autoimmunity in a Murine Lupus Model of Peptide-Induced Immune Tolerance Depends on FoxP3 Expression. *J Immunol* (2007) 178:7649–57. doi: 10.4049/jimmunol.178.12.7649
36. Hahn BH, Anderson M, Le E, La Cava A. Anti-Dna Ig Peptides Promote Treg Cell Activity in Systemic Lupus Erythematosus Patients. *Arthritis Rheum* (2008) 58:2488–97. doi: 10.1002/art.23609
37. Dinesh R, Hahn BH, La Cava A, Singh RP. Interferon-Inducible Gene 202b Controls CD8(+) T Cell-Mediated Suppression in Anti-DNA Ig Peptide-Treated (NZB X NZW) F1 Lupus Mice. *Genes Immun* (2011) 12:360–9. doi: 10.1038/gene.2011.4
38. Brunkow ME, Jeffery EW, Hjerrild KA, Paeper B, Clark LB, Yasayko SA, et al. Disruption of a New Forkhead/Winged-Helix Protein, Scurfin, Results in the Fatal Lymphoproliferative Disorder of the Scurfy Mouse. *Nat Genet* (2001) 27:68–73. doi: 10.1038/83784
39. Fontenot JD, Gavin MA, Rudensky AY. FoxP3 Programs the Development and Function of CD4+CD25+ Regulatory T Cells. *Nat Immunol* (2003) 4:330–6. doi: 10.1038/ni904
40. Fontenot JD, Rasmussen JP, Williams LM, Dooley JL, Farr AG, Rudensky AY. Regulatory T Cell Lineage Specification by the Forkhead Transcription Factor FoxP3. *Immunity* (2005) 22:329–41. doi: 10.1016/j.immuni.2005.01.016
41. 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
42. Marson A, Kretschmer K, Frampton GM, Jacobsen ES, Polansky JK, MacIsaac KD, et al. FoxP3 Occupancy and Regulation of Key Target Genes During T-cell Stimulation. *Nature* (2007) 445:931–5. doi: 10.1038/nature05478
43. Tsujimoto Y, Finger LR, Yunis J, Nowell PC, Croce CM. Cloning of the Chromosome Breakpoint of Neoplastic B Cells With the T(14;18) Chromosome Translocation. *Science* (1984) 226:1097–9. doi: 10.1126/science.6093263
44. Cleary ML, Smith SD, Sklar J. Cloning and Structural Analysis of cDNAs for Bcl-2 and a Hybrid bcl-2/immunoglobulin Transcript Resulting From the T (14;18) Translocation. *Cell* (1986) 47:19–28. doi: 10.1016/0092-8674(86)90362-4
45. Blair PA, Norena LY, Flores-Borja F, Rawlings DJ, Isenberg DA, Ehrenstein MR, et al. CD19(+)CD24(Hi)CD38(Hi) B Cells Exhibit Regulatory Capacity in Healthy Individuals But are Functionally Impaired in Systemic Lupus Erythematosus Patients. *Immunity* (2010) 32:129–40. doi: 10.1016/j.immuni.2009.11.009
46. 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
47. Flores-Borja F, Bosma A, Ng D, Reddy V, Ehrenstein MR, Isenberg DA, et al. Cd19+Cd24hicd38hi B Cells Maintain Regulatory T Cells While Limiting TH1 and TH17 Differentiation. *Sci Trans Med* (2013) 5:173ra23. doi: 10.1126/scitranslmed.3005407
48. Lourenco EV, Procaccini C, Ferrera F, Iikuni N, Singh RP, Filaci G, et al. Modulation of P38 MAPK Activity in Regulatory T Cells After Tolerance With Anti-DNA Ig Peptide in (NZB X NZW)F1 Lupus Mice. *J Immunol* (2009) 182:7415–21. doi: 10.4049/jimmunol.0804214
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. 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. 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
52. Jury EC, Flores-Borja F, Kalsi HS, Lazarus M, Isenberg DA, Mauri C, et al. Abnormal CTLA-4 Function in T Cells From Patients With Systemic Lupus Erythematosus. *Eur J Immunol* (2010) 40:569–78. doi: 10.1002/eji.200939781
53. Liu J, Zhang HX. CTLA-4 Polymorphisms and Systemic Lupus Erythematosus: A Comprehensive Meta-Analysis. *Genet Test Mol Biomarkers* (2013) 17:226–31. doi: 10.1089/gtmb.2012.0302
54. Choubey D, Panchanathan R. Interferon-Inducible Ifi200-family Genes in Systemic Lupus Erythematosus. *Immunol Lett* (2008) 119:32–41. doi: 10.1016/j.imlet.2008.06.001
55. Crow MK. Type I Interferon in the Pathogenesis of Lupus. *J Immunol* (2014) 192:5459–68. doi: 10.4049/jimmunol.1002795
56. Ronnblom L, Pascual V. The Innate Immune System in SLE: Type I Interferons and Dendritic Cells. *Lupus* (2008) 17:394–9. doi: 10.1177/0961203308090020
57. Luo S, Wang Y, Zhao M, Lu Q. The Important Roles of Type I Interferon and Interferon-Inducible Genes in Systemic Lupus Erythematosus. *Int Immunopharmacol* (2016) 40:542–9. doi: 10.1016/j.intimp.2016.10.012
58. Bezalel S, Guri KM, Elbirt D, Asher I, Sthoeger ZM. Type I Interferon Signature in Systemic Lupus Erythematosus. *Isr Med Assoc J* (2014) 16:246–9.
59. Crow MK. Type I Interferon in Systemic Lupus Erythematosus. *Curr Top Microbiol Immunol* (2007) 316:359–86. doi: 10.1007/978-3-540-71329-6_17
60. Crow MK. Type I Interferon in Organ-Targeted Autoimmune and Inflammatory Diseases. *Arthritis Res Ther* (2010) 12(Suppl 1):S5. doi: 10.1186/ar2886
61. Crow MK. Advances in Understanding the Role of Type I Interferons in Systemic Lupus Erythematosus. *Curr Opin Rheumatol* (2014) 26:467–74. doi: 10.1097/BOR.0000000000000087
62. Sthoeger Z, Zinger H, Sharabi A, Asher I, Mozes E. The Tolerogenic Peptide, hCDR1, Down-Regulates the Expression of Interferon-Alpha in Murine and Human Systemic Lupus Erythematosus. *PloS One* (2013) 8:e60394. doi: 10.1371/journal.pone.0060394
63. Baechler EC, Batliwalla FM, Karypis G, Gaffney PM, Ortmann WA, Espe KJ, et al. Interferon-Inducible Gene Expression Signature in Peripheral Blood Cells of Patients With Severe Lupus. *Proc Natl Acad Sci USA* (2003) 100:2610–5. doi: 10.1073/pnas.0337679100
64. Crow MK, Kirou KA. Interferon-Alpha in Systemic Lupus Erythematosus. *Curr Opin Rheumatol* (2004) 16:541–7. doi: 10.1097/01.bor.0000135453.70424.1b
65. Ytterberg SR, Schnitzer TJ. Serum Interferon Levels in Patients With Systemic Lupus Erythematosus. *Arthritis Rheum* (1982) 25:401–6. doi: 10.1002/art.1780250407

66. Liu Z, Davidson A. Ifnalpha Inducible Models of Murine Sle. *Front Immunol* (2013) 4:306. doi: 10.3389/fimmu.2013.00306
67. Domeier PP, Chodisetti SB, Schell SL, Kawasawa YI, Fasnacht MJ, Soni C, et al. B-Cell-Intrinsic Type 1 Interferon Signaling Is Crucial for Loss of Tolerance and the Development of Autoreactive B Cells. *Cell Rep* (2018) 24:406–18. doi: 10.1016/j.celrep.2018.06.046
68. Kiefer K, Oropallo MA, Cancro MP, Marshak-Rothstein A. Role of Type I Interferons in the Activation of Autoreactive B Cells. *Immunol Cell Biol* (2012) 90:498–504. doi: 10.1038/icb.2012.10
69. Celhar T, Fairhurst AM. Toll-Like Receptors in Systemic Lupus Erythematosus: Potential for Personalized Treatment. *Front Pharmacol* (2014) 5:265. doi: 10.3389/fphar.2014.00265
70. Fillatreau S, Manfroi B, Dorner T. Toll-Like Receptor Signalling in B Cells During Systemic Lupus Erythematosus. Nature Reviews. *Rheumatology* (2020) 17, 98-108. doi: 10.1038/s41584-020-00544-4
71. Khamashta M, Merrill JT, Werth VP, Furie R, Kalunian K, Illei GG, et al. Sifalimumab, an Anti-Interferon-Alpha Monoclonal Antibody, in Moderate to Severe Systemic Lupus Erythematosus: A Randomised, Double-Blind, Placebo-Controlled Study. *Ann Rheum Dis* (2016) 75:1909–16. doi: 10.1136/annrheumdis-2015-208562
72. Kalunian KC, Merrill JT, Maciuca R, McBride JM, Townsend MJ, Wei X, et al. A Phase II Study of the Efficacy and Safety of Rontalizumab (rhuMAb Interferon-Alpha) in Patients With Systemic Lupus Erythematosus (ROSE). *Ann Rheum Dis* (2016) 75:196–202. doi: 10.1136/annrheumdis-2014-206090
73. Chaichian Y, Wallace DJ, Weisman MH. A Promising Approach to Targeting Type 1 IFN in Systemic Lupus Erythematosus. *J Clin Invest* (2019) 129:958–61. doi: 10.1172/JCI127101
74. Singh RR, Ebling FM, Sercarz EE, Hahn BH. Immune Tolerance to Autoantibody-Derived Peptides Delays Development of Autoimmunity in Murine Lupus. *J Clin Invest* (1995) 96:2990–6. doi: 10.1172/JCI118371
75. Waisman A, Ruiz PJ, Israeli E, Eilat E, Konen-Waisman S, Zinger H, et al. Modulation of Murine Systemic Lupus Erythematosus With Peptides Based on Complementarity Determining Regions of a Pathogenic anti-DNA Monoclonal Antibody. *Proc Natl Acad Sci USA* (1997) 94:4620–5. doi: 10.1073/pnas.94.9.4620
76. Sthoeger ZM, Dayan M, Tcherniack A, Green L, Toledo S, Segal R, et al. Modulation of Autoreactive Responses of Peripheral Blood Lymphocytes of Patients With Systemic Lupus Erythematosus by Peptides Based on Human and Murine anti-DNA Autoantibodies. *Clin Exp Immunol* (2003) 131:385–92. doi: 10.1046/j.1365-2249.2003.02058.x
77. Sharabi A, Haviv A, Zinger H, Dayan M, Mozes E. Amelioration of Murine Lupus by a Peptide, Based on the Complementarity Determining Region-1 of an Autoantibody as Compared to Dexamethasone: Different Effects on Cytokines and Apoptosis. *Clin Immunol* (2006) 119:146–55. doi: 10.1016/j.clim.2006.01.007
78. Sharabi A, Zinger H, Zborowsky M, Sthoeger ZM, Mozes E. A Peptide Based on the Complementarity-Determining Region 1 of an Autoantibody Ameliorates Lupus by Up-Regulating CD4+CD25+ Cells and TGF-Beta. *Proc Natl Acad Sci USA* (2006) 103:8810–5. doi: 10.1073/pnas.0603201103
79. Sela U, Mauermann N, Hershkoviz R, Zinger H, Dayan M, Cahalon L, et al. The Inhibition of Autoreactive T Cell Functions by a Peptide Based on the CDR1 of an anti-DNA Autoantibody is Via TGF-beta-mediated Suppression of LFA-1 and CD44 Expression and Function. *J Immunol* (2005) 175:7255–63. doi: 10.4049/jimmunol.175.11.7255
80. Urowitz MB, Isenberg DA, Wallace DJ. Safety and Efficacy of hCDR1 (Edratide) in Patients With Active Systemic Lupus Erythematosus: Results of Phase II Study. *Lupus Sci Med* (2015) 2:e000104. doi: 10.1136/lupus-2015-000104
81. Plater-Zyberk C, Taylor PC, Blaylock MG, Maini RN. Anti-CD5 Therapy Decreases Severity of Established Disease in Collagen Type II-induced Arthritis in DBA/1 Mice. *Clin Exp Immunol* (1994) 98:442–7. doi: 10.1111/j.1365-2249.1994.tb05510.x
82. Vadasz Z, Peri R, Eiza N, Slobodin G, Balbir-Gurman A, Toubi E. The Expansion of CD25 High IL-10 High FoxP3 High B Regulatory Cells is in Association With SLE Disease Activity. *J Immunol Res* (2015) 2015:254245. doi: 10.1155/2015/254245
83. Noh J, Choi WS, Noh G, Lee JH. Presence of FoxP3-expressing Cd19(+)Cd5(+) B Cells in Human Peripheral Blood Mononuclear Cells: Human CD19(+) CD5(+)FoxP3(+) Regulatory B Cell (Breg). *Immune Netw* (2010) 10:247–9. doi: 10.4110/in.2010.10.6.247
84. Parameswaran R, Ben David H, Sharabi A, Zinger H, Mozes E. B-Cell Activating Factor (BAFF) Plays a Role in the Mechanism of Action of a Tolerogenic Peptide That Ameliorates Lupus. *Clin Immunol* (2009) 131:223–32. doi: 10.1016/j.clim.2008.12.009
85. Lapter S, Ben-David H, Sharabi A, Zinger H, Telerman A, Gordin M, et al. A Role for the B-cell CD74/Macrophage Migration Inhibitory Factor Pathway in the Immunomodulation of Systemic Lupus Erythematosus by a Therapeutic Tolerogenic Peptide. *Immunology* (2011) 132:87–95. doi: 10.1111/j.1365-2567.2010.03342.x
86. La Cava A. Targeting the BLyS-APRIL Signaling Pathway in SLE. *Clin Immunol* (2013) 148:322–7. doi: 10.1016/j.clim.2012.11.010
87. Stohl W. Therapeutic Targeting of the BAFF/APRIL Axis in Systemic Lupus Erythematosus. *Expert Opin Ther Targets* (2014) 18:473–89. doi: 10.1517/14728222.2014.888415
88. Stohl W. Inhibition of B Cell Activating Factor (BAFF) in the Management of Systemic Lupus Erythematosus (SLE). *Expert Rev Clin Immunol* (2017) 13:623–33. doi: 10.1080/1744666X.2017.1291343
89. Ben-David H, Sharabi A, Parameswaran R, Zinger H, Mozes E. A Tolerogenic Peptide Down-Regulates Mature B Cells in Bone Marrow of Lupus-Afflicted Mice by Inhibition of interleukin-7, Leading to Apoptosis. *Immunology* (2009) 128:245–52. doi: 10.1111/j.1365-2567.2009.03109.x
90. Sharabi A, Luger D, Ben-David H, Dayan M, Zinger H, Mozes E. The Role of Apoptosis in the Ameliorating Effects of a CDR1-based Peptide on Lupus Manifestations in a Mouse Model. *J Immunol* (2007) 179:4979–87. doi: 10.4049/jimmunol.179.8.4979
91. Strasser A, Whittingham S, Vaux DL, Bath ML, Adams JM, Cory S, et al. Enforced BCL2 Expression in B-lymphoid Cells Prolongs Antibody Responses and Elicits Autoimmune Disease. *Proc Natl Acad Sci USA* (1991) 88:8661–5. doi: 10.1073/pnas.88.19.8661

Conflict of Interest: BHH and RPS have a patent through the University of California, Los Angeles for the use of pCons as an immune modulator in systemic lupus erythematosus.

The remaining 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.

frontiers in Immunology

ORIGINAL RESEARCH
published: 26 May 2021
doi: 10.3389/fimmu.2021.628059

Nanoparticles Engineered as Artificial Antigen-Presenting Cells Induce Human CD4$^+$ and CD8$^+$ Tregs That Are Functional in Humanized Mice

Sophia Giang[1], *David A. Horwitz*[2,3]*, *Sean Bickerton*[4] *and Antonio La Cava*[1]*

[1] Department of Medicine, University of California Los Angeles, Los Angeles, CA, United States, [2] General Nanotherapeutics, Santa Monica, CA, United States, [3] Keck School of Medicine, University of Southern California, Los Angeles, CA, United States, [4] Department of Biomedical Engineering, Yale University, New Haven, CT, United States

OPEN ACCESS

Edited by:
Herman Waldmann,
University of Oxford, United Kingdom

Reviewed by:
Marcus O. Butler,
University Health Network, Canada
Paul Anthony Gleeson,
The University of Melbourne, Australia
Ursula Grohmann,
University of Perugia, Italy

***Correspondence:**
Antonio La Cava
alacava@mednet.ucla.edu
David A. Horwitz
dhorwitz@usc.edu

Specialty section:
This article was submitted to Immunological Tolerance and Regulation, a section of the journal Frontiers in Immunology

Received: 10 November 2020
Accepted: 13 April 2021
Published: 26 May 2021

Citation:
Giang S, Horwitz DA, Bickerton S and La Cava A (2021) Nanoparticles Engineered as Artificial Antigen-Presenting Cells Induce Human CD4$^+$ and CD8$^+$ Tregs That Are Functional in Humanized Mice. Front. Immunol. 12:628059. doi: 10.3389/fimmu.2021.628059

Artificial antigen-presenting cells (aAPCs) are synthetic versions of naturally occurring antigen-presenting cells (APCs) that, similar to natural APCs, promote efficient T effector cell responses *in vitro*. This report describes a method to produce acellular tolerogenic aAPCs made of biodegradable poly lactic-co-glycolic acid (PLGA) nanoparticles (NPs) and encapsulating IL-2 and TGF-β for a paracrine release to T cells. We document that these aAPCs can induce both human CD4$^+$ and CD8$^+$ T cells to become FoxP3$^+$ T regulatory cells (Tregs). The aAPC NP-expanded human Tregs are functional *in vitro* and can modulate systemic autoimmunity *in vivo* in humanized NSG mice. These findings establish a proof-of-concept to use PLGA NPs as aAPCs for the induction of human Tregs *in vitro* and *in vivo*, highlighting the immunotherapeutic potential of this targeted approach to repair IL-2 and/or TGF-β defects documented in certain autoimmune diseases such as systemic lupus erythematosus.

Keywords: autoimmunity, T cells, T regulatory cells, immune tolerance, systemic lupus erythematosus, graft-versus-host disease, nanoparticles, humanized mice

INTRODUCTION

In natural conditions, the fate of activated T cells is determined by interaction between antigen-presenting cells (APCs) and T cells. This involves the engagement of the MHC/peptide complex on the APC and the T-cell receptor (TCR) on the T cell, and the complementary costimulatory molecules on the APC and the T cell. The modulation of this critical step of the adaptive immune response can have very important immunotherapeutic implications in the clinic for the generation of effector and regulatory immune responses. However, the fine tuning of APCs interactions with T cells for therapeutic purposes has been difficult to achieve. The natural sources of APCs are scarce. Large amounts of starting cells are required for enrichment and/or sorting, and technical procedures for their preparation are costly and time-intensive. For these reasons, alternative procedures have been developed that include engineering cellular and acellular artificial APCs (aAPCs) (1). Cellular aAPCs are engineered from primary cells or from transformed (human or xenogeneic) cells using

retroviral or lentiviral vectors to express the desired costimulatory molecules and adhesion molecules for the expansion and/or long-term growth of functional T cells (2). Other cellular aAPCs may also express human HLA molecules to generate antigen-specific cells for patients with a given HLA (3). Those cellular aAPCs that carry the necessary components for interaction and engagement of the cell surface ligands on T cells for activation and proliferation (4, 5) have been used in cancer immunotherapy and immunization in infection because of their ability to generate effector T cell responses. However, their pro-inflammatory activity makes them unsuitable in settings where effector immune responses are deleterious to the host, such as in autoimmunity.

We report here the development of acellular aAPCs that target T cells and induce them to become functional Tregs *in vitro* and *in vivo*. We had previously shown that nanoparticle (NP)-mediated delivery of IL-2 and TGF-β (two cytokines that are deficient in SLE) to mouse $CD2^+$ and $CD4^+$ cells induced tolerogenic immune responses that protected mice from a lupus-like syndrome (6, 7). Here we extend those results and show that we can induce human functional $CD4^+$ and $CD8^+$ Tregs that suppress xenogenic graft-versus-host disease (GvHD) in humanized mice using aAPC NPs, providing a proof-of-principle of immunotherapeutic restoration of immune homeostasis in conditions of immune dysregulation associated with chronic inflammation.

METHODS

Preparation of PLGA Nanoparticles

Poly lactic-co-glycolic acid (PLGA) NPs were prepared as described elsewhere (6). After preparation, the NPs were characterized through examination of physical properties, encapsulation metrics, and release kinetics according to standard procedures (6). By dynamic light scattering, NPs were found to have a mean ± SD hydrodynamic diameter of 245 ± 2 nm with a low polydispersity index indicative of a uniform NP population with a relatively tight size distribution. Cytokine encapsulation was measured by ELISA after NPs were disrupted using DMSO, and standard curves were generated using cytokine standards with all wells supplemented to contain 5% volume/volume DMSO and the appropriate concentration of empty NPs. NPs contained a mean ± SD of 7.4 ± 0.4 ng TGF-β and 1.9 ± 0.1 ng IL-2 per mg of NP. For cell targeting, NPs diluted in PBS were incubated 10 minutes prior to use with the relevant biotinylated targeting antibody (anti-CD4, -CD8 or -CD3) at a concentration ratio of 2 μg antibody/mg NP.

Preparation of Human PBMCs

Human peripheral blood mononuclear cells (PBMCs) were prepared from heparinized venous blood of healthy adult volunteers by Ficoll-Hypaque density gradient centrifugation and used fresh (for transfer experiments) or cultured for 5 days in U-bottom well plates at a concentration of 0.5 x 10^6/well in complete AIM V™ medium (Thermo Fisher Scientific, Waltham, MA). All protocols that involved human blood donors were approved by the IRB at the University of California Los Angeles. In some experiments, PBMCs were cultured with anti-human CD3/CD28 Dynabeads (Thermo Fisher Scientific) or with IL-2 (100 U/ml) and TGF-β (5 ng/ml) or anti-TGF-β (1D11) (all from R&D Systems, Minneapolis, MN). *In vitro* suppression assays were performed according to standard protocols (6). $CD4^+CD25^-$ T cells isolated by negative selection to a purity of >95% using the Miltenyi Biotec $CD4^+CD25^+CD127^{dim/-}$ Regulatory T Cell Isolation kit II served as responder cells in cocultures for 3 days with autologous Tregs (positive fraction) isolated with the same kit, following the manufacturer's instructions. Culture supernatants were analyzed for IFN-γ content by ELISA (R&D Systems). Proliferation was evaluated by a liquid scintillation counter following addition of ^{3}H-thymidine (1 μCi/well) 16 hours before analysis.

Flow Cytometry

Human PBMCs or magnetic-bead sorted cells were stained following standard procedures with the following FITC-, PE-, PerCP- or APC-conjugated anti-human antibodies: CD4 (RPA-T4), CD8 (RPA-T8), CD25 (MEM-181), CD127 (eBioRDR5), FoxP3 (PCH101), CD122 (TU27), CD45RA (HI100), or isotype controls. All antibodies were from Thermo Fisher Scientific. Data were acquired on a FACSCalibur™ flow cytometer (BD Biosciences, San Jose, CA) and analyzed using FlowJo™ software (BD, Franklin Lakes, NJ).

Mice

To assess the functional properties of the human Tregs induced by aAPCs, we used the human-anti-mouse xenogeneic GvHD model. The disease develops in recipient NOD/*scid*/*IL2r* common γ chain$^{-/-}$ (NSG) mice following the transfer of human PBMCs (8) and, like human lupus, these mice develop B cell hyperactivity and increased IgG production. NSG mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and housed under specific pathogen-free conditions in microisolator cages with unrestricted access to autoclaved food and sterile water. 10^7 fresh human PBMCs were resuspended in 200 μl of PBS in insulin syringes and injected i.v. *via* the tail vein into individual unconditioned NSG mice of 8-12 weeks of age. The mice also received i.v. (individually) 1.5 mg IL-2/TGF-β-loaded NPs decorated with anti-CD3 (OKT3, Thermo Fisher Scientific), starting on the day of transfer of human PBMCs, according to a previously described protocol (6): day 0, 3, 6, 9, 12. Control mice received empty uncoated NPs or PBS under identical conditions as the above NP-treated mice. The experiments were performed according to the guidelines of the Institutional Animal Committee of the University of California Los Angeles. Animals that developed hunched posture combined with lethargy and/or lack of grooming, reduced mobility or tachypnea, were euthanized and an end-point of survival was recorded at the time of sacrifice. Disease was monitored using a validated scoring system (9) that evaluates each of the five following parameters as 0 if absent or 1 if present: 1) weight loss >10% of initial weight; 2) hunching posture; 3) skin lesions (patchy alopecia); 4) dull fur; 5) diarrhea. Dead mice received a

total score of 5 until the end of experiment. Peripheral blood (to separate PBMCs for flow cytometry) and plasma were collected on days 0, 4, 14, 21 and 50. Plasma concentrations of human IgG were measured by ELISA (Thermo Fisher Scientific). For histologic evaluations, lung, liver and colon were collected on day 50 after the transfer of PBMCs. Tissues were fixed in formalin, paraffin embedded, and sections stained with hematoxylin/eosin.

Statistical Analyses

Assessment for normal distribution was done by Shapiro-Wilks test. Comparisons between two groups were evaluated using (post-hoc) Student's *t* test; comparisons among multiple groups used one-way ANOVA with Bonferroni's correction. Differences in Kaplan-Meier survival curves were analyzed by the log-rank test. Data were analyzed using GraphPad Prism software; *P* values of <0.05 were considered significant.

RESULTS

Use of NPs as Acellular aAPCs to Induce $CD4^+CD25^{hi}FoxP3^+CD127^-$ and $CD8^+FoxP3^+$ T Cells

We recently reported that NPs loaded with IL-2 and TGF-β and targeted to T cells inhibited the production of anti-DNA autoantibodies in (C57BL/6 × DBA/2)F_1 (BDF1) hybrid mice that develop lupus-like disease following the transfer of splenocytes from parental DBA/2 mice (6). Specifically, the NPs promoted a switch to tolerogenic responses with an induction of $CD4^+$ and $CD8^+$ Tregs that were responsible for the mitigation of disease manifestations and prolonged the survival of BDF1 lupus mice.

To extend those findings to humans, PBMCs from healthy donors were incubated with PLGA NPs loaded with IL-2 and TGF-β and targeted to $CD4^+$ and $CD8^+$ T cells. The observed expansion *in vitro* of $CD4^+CD25^{hi}FoxP3^+CD127^-$ (**Figure 1A**) and $CD8^+FoxP3^+$ T cells (**Figure 1B**) after NP-mediated delivery of tolerogenic cytokines to human $CD4^+$ and $CD8^+$ T cells suggested the induction of immunoregulatory cells (10–14).

Paracrine Delivery of Cytokines to Human T Cells by aAPC NPs Leads to the Induction and Expansion of Functional Tregs

These findings led us to wonder whether the NPs could induce a tolerogenic T-cell program by acting as acellular aAPCs that delivered engage the TCR rather than CD4 or CD8 and deliver IL-2 and TGF-β. We found that the NPs loaded with tolerogenic cytokines and coated with anti-CD3/28 antibodies to trigger TCR stimulation efficiently expanded $CD4^+CD25^{hi}FoxP3^+CD127^-$ (**Figure 1A**) and $CD8^+FoxP3^+$ (**Figure 1B**) T cells *in vitro*, indicating the ability of NPs to operate as acellular aAPCs capable to induce human T cells with an immunoregulatory phenotype.

Having found that the delivery of IL-2 and TGF-β to T cells by the NPs allowed human T cell differentiation into $CD4^+CD25^{hi}FoxP3^+CD127^-$ and $CD8^+FoxP3^+$ T cells, we investigated the temporal contribution of TGF-β to the process. The presence of TGF-β was required for the induction of $CD4^+$ and $CD8^+$ Tregs but was dispensable for their expansion since its blockade did not influence expansion (**Figure 2A**). Both IL-2 and TGF-β were required for the induction but IL-2 alone could promote expansion (**Figure 2B**). Thus, the IL-2 and TGF-β delivered by the aAPC NPs play different roles in the generation of the human Tregs, being both required for induction but being only IL-2 required for expansion.

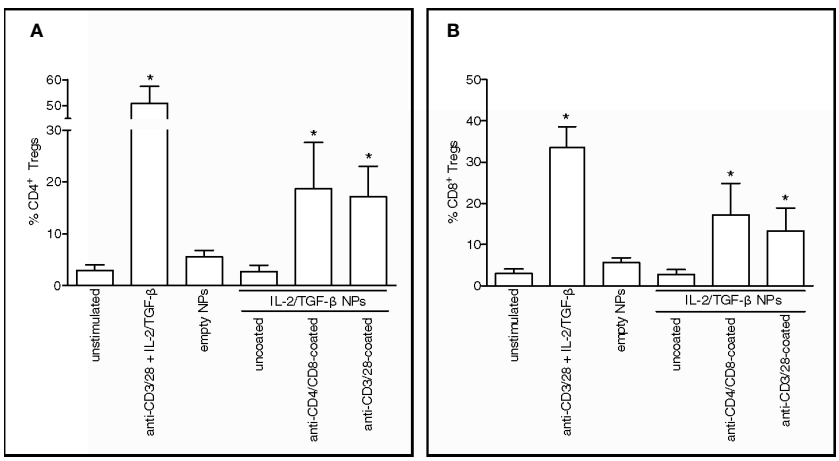


FIGURE 1 | Nanoparticles (NPs) loaded with tolerogenic cytokines and targeted to human T cells induce $CD4^+$ and $CD8^+$ Tregs *in vitro*. PBMCs from healthy volunteers (n = 5) were cultured for 5 days in the presence of 100 μg/ml NPs loaded with IL-2 and TGF-β that had been either left uncoated or decorated with antibodies to T cells (anti-CD4/CD8 or anti-CD3/28). Cultures with medium only and either no NPs (unstimulated) or NPs kept unloaded (empty) served as negative controls; cultures with anti-CD3/28 beads at a ratio of 0.2 beads/cell in the presence of soluble IL-2 and TGF-β served as positive control. Results show increased numbers of $CD4^+CD25^{hi}CD127^-FoxP3^+$ **(A)** and $CD8^+FoxP3^+$ T cells **(B)** in the presence of NPs decorated with anti-CD4/CD8 Ab or anti-CD3/CD28 Ab. *P < 0.05 by the Student's *t* test in the comparison with unstimulated cells.

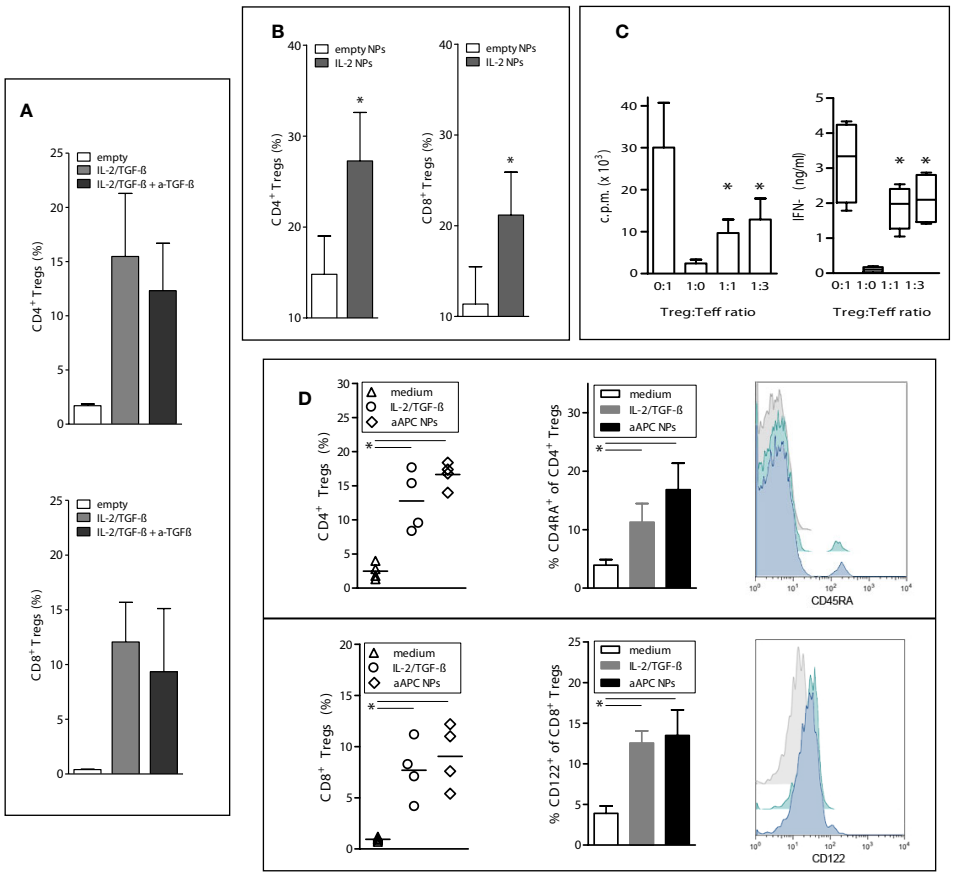


FIGURE 2 | Paracrine delivery of cytokines by aAPC NPs to human T cells favors the induction and expansion of functional Tregs *in vitro*. To induce Tregs, human PBMCs from healthy donors (n = 6) were cultured with IL-2/TGF-β-loaded NPs targeted to T cells (decorated with anti-CD3/28) for 5 days. To study the expansion of the induced Tregs, the cultured cells were then washed twice before incubation with IL-2-encapsulating NPs or empty NPs for 5 more days with or without anti-TGF-β antibody (10 μg/ml). **(A)** The expansion of aAPC NP-induced $CD4^+$ and $CD8^+$ Tregs is not influenced significantly by the blockade of TGF-β. P not significant in the presence or absence of anti-TGF-β. **(B)** T-cell-targeted NPs that only encapsulate IL-2 promote the expansion of $CD4^+$ and $CD8^+$ Tregs; *P < 0.05 by the Student's *t* test. **(C)** $CD4^+$ Tregs induced by aAPC NPs targeted to T cells suppress *in vitro* the proliferation (left) and IFN-γ production (right) of cocultured $CD4^+CD25^-$ T cells. *P < 0.05 in the comparison with Treg : Teff at the 0:1 ratio (only stimulated T effector cells). **(D)** Comparison of induction of $CD4^+$ (top) and $CD8^+$ (bottom) Tregs by aAPC NPs decorated with anti-CD3/28 vs. anti-CD3/28 beads in the presence of soluble IL-2/TGF-β. Control cultures had medium only. Percentage numbers and representative histograms (grey, medium only; green, IL-2/TGF-β; blue, aAPC NPs) for the expression of CD45RA by $CD4^+$ Tregs and CD122 by $CD8^+$ Tregs (n = 4 donors). *P < 0.05 by the Student's *t* test in the comparison vs. medium only; not significant between cultures with IL-2/TGF-β and aAPC NPs.

The activity of the aAPC NP-induced cells was confirmed by assays of *in vitro* suppression that indicated that the aAPC NP-induced $CD4^+$ Tregs efficiently suppressed proliferation and production of proinflammatory cytokines from T effector cells (**Figure 2C**). Of note, aAPC NPs induced similar numbers of both $CD4^+$ and $CD8^+$ Tregs (10–14) as compared to those standardly induced by incubation with IL-2 and TGF-β (6, 15) (**Figure 2D**). Moreover, the incubation with aAPC NPs increased the expression of CD122 on $CD8^+FoxP3^+$ cells and CD45RA on $CD4^+FoxP3^+$ cells (**Figure 2D**). The interest of this finding lies in the fact that CD122 is a classical $CD8^+$ Treg marker (15), while $CD45RA^+$ $CD4^+$ Tregs are valuable for adoptive transfer of $CD4^+$ Tregs in immune-mediated disorders since they maintain FoxP3 expression and retain homing receptors (CD62 and CCR7) after extensive proliferation (16).

Induction of Tregs *In Vivo* by aAPC NPs Associates With the Protection of Humanized NSG Mice From Lupus-Like Disease

Since the suppressive activity of the Tregs *in vitro* might not necessarily correlate with a suppressive activity *in vivo* (17), we evaluated the relevance of the above *in vitro* findings to *in vivo* settings. Taking advantage of the known protective effects of Tregs in allograft rejection, we tested the immunotherapeutic potential of the aAPC NPs in a mouse model of human-anti-mouse GvHD (which reproduces manifestations of lupus-like disease *in vivo*) (8). Individual NSG mice received i.v. 10^7 human PBMCs to develop GvHD. One group concomitantly received (anti-CD3 Ab-) T-cell targeted NPs encapsulating IL-2/TG-β, one control group received empty uncoated NPs, and another control group only received vehicle (PBS). The results showed

that the mice that received T-cell targeted NPs encapsulating IL-2/TG-β had an *in vivo* expansion of both $CD4^+$ and $CD8^+$ Tregs that was absent in mice that had received empty NPs or no NPs (**Figure 3A**) and that associated with a reduction in the levels of circulating human IgG (**Figure 3B**). Of note, the expansion of the Tregs remained above baseline levels throughout the experiment until its termination at day 50 (**Figure 3A**).

Importantly, the NSG mice that received T-cell targeted NPs loaded with IL-2/TGF-β had significantly reduced disease manifestations. The aAPC NP-protected mice did not lose weight after the transfer of human PBMCs (**Figure 4A**) and had an extended survival (**Figure 4B**) as compared to the mice that had not received NPs or that had received empty NPs (**Figures 4A, B**). Mice treated with NPs also had reduced human IgG levels (**Figure 4C**) and improved skin morphology (**Figure 4D**). Finally, the histopathology of lung, liver and colon of the NSG mice receiving aAPC NPs showed a significant protection as compared to the control mice (**Figure 4E**). These results provide evidence that the aAPC NPs induced therapeutic Tregs.

DISCUSSION

We show that PLGA NPs can be used as acellular aAPCs for *in vitro* and *in vivo* expansion of functional human Tregs that suppress xenogeneic graft-versus-host disease. In the *in vitro* experiments with human cells, the aAPC NPs containing IL-2 and TGF-β were decorated with anti-CD3/CD28. In the *in vivo* experiments, the NPs were decorated with anti-CD3 Ab only since human T cells were activated by the mouse MHC antigens. In both cases $CD4^+CD25^{hi}CD127^{lo}FoxP3^+$ and $CD8^+FoxP3^+$ Tregs were generated and expanded. While IL-2 and TGF-β were required for Treg induction, only IL-2 was required for Tregs expansion.

When APCs engage the TCR through the MHC/antigen complex and provide costimulatory signals to T lymphocytes, cell differentiation and functional activation ensue. The replication of this process by aAPCs - used as synthetic platforms - can recapitulate the natural interaction between APCs and T cells, allowing the delivery of signals to T cells and the initiation of adaptive immune responses (5, 18) that include a paracrine delivery of IL-2 to T cells (as in our aAPCs). Our strategy of employing aAPCs that encapsulated a payload for the promotion of tolerogenic immune responses could represent a new tool with immunotherapeutic potential, being effective in humanized mice. The fact that PLGA is biocompatible and has a favorable safety profile in clinical settings envisions the possibility of a rapid translational potential of this approach to the clinic (19).

The expansion of human Tregs with aAPC NPs has the advantage of limiting the deleterious effects associated with an *in vivo* induction of Tregs achievable through systemic treatments with cytokines that carry non-targeted actions. We acknowledge that our NPs did not include components of antigen specificity, differently from the paramagnetic iron-dextran NPs that expressed peptide/MHC and anti-CD28 antibodies and were used in organ-specific autoimmune diseases (20, 21). We believe that the induction of polyclonal Tregs might be advantageous in conditions such as SLE, where the chronic systemic autoimmune response to multiple self-antigens (22) benefits from polyclonal Tregs (23). We also think that the expansion of both $CD4^+$ and $CD8^+$ Tregs *in vivo* in SLE will have more beneficial effects than that of each subset alone because of the protective activity of $CD8^+$ Tregs in the disease (15). However, a limitation of our study is that it does not distinguish the relative contribution of the $CD4^+$ and $CD8^+$ Tregs in the observed *in vivo* protective effects. Since in previous work with $CD4^+$ and $CD8^+$ Tregs induced *ex vivo* the $CD8^+$ Tregs were major contributors to the protection of immunodeficient mice from human anti-mouse GvHD through non-cytotoxic suppressive effects on allogeneic cells (24), we suggest that the $CD8^+$ Tregs in our current study did contribute to the protective effects on the human anti-mouse GvHD. The extent needs to be investigated directly.

Multiple possible therapeutic applications can benefit from the utilization of Tregs yet several problems hamper practical

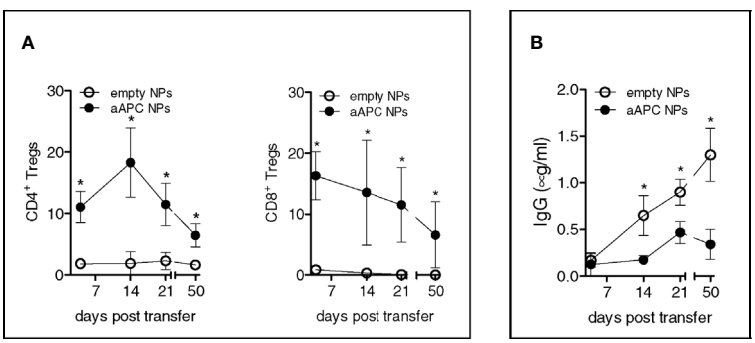


FIGURE 3 | Humanized NSG mice treated with aAPC NPs have increased numbers of circulating $CD4^+$ and $CD8^+$ Tregs and reduced levels of human IgG. Individual NSG mice (n = 12) that received i.v. 10^7 human PBMCs each for the induction of lupus-like disease were divided into two groups of 6 mice each. One group received IL-2/TGF-β-loaded NPs targeted to T cells (decorated with anti-CD3 Ab), the other group that served as control received empty, untargeted NPs according to the protocol detailed in the *Methods*. **(A)** PBMCs were analyzed *ex vivo* by flow cytometry at the time points indicated. Relative frequency of peripheral $CD4^+$ and $CD8^+$ Tregs was derived from counts of $CD4^+$ and $CD8^+$ T cells. **(B)** Human IgG antibodies in plasma were measured by ELISA. *P < 0.05 by the Student's *t* test.

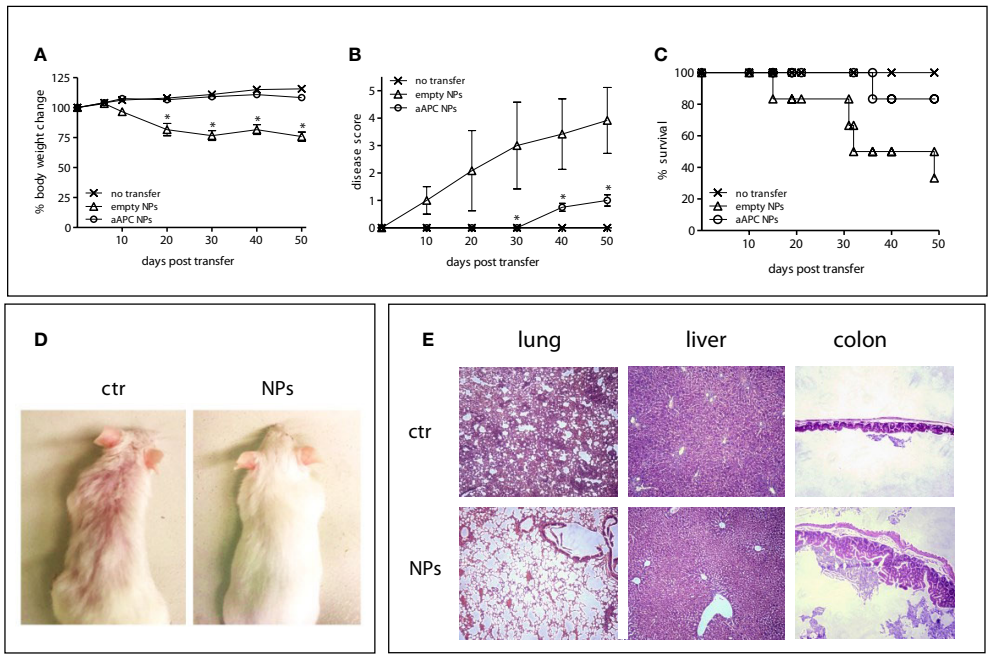


FIGURE 4 | aAPC NPs engineered to induce Tregs *in vivo* suppress lupus-like disease in NSG mice transferred with xenogenic human PBMCs. Six NSG mice per group were left untreated (no transfer) or injected with human PBMCs together with PBS (ctr) or aAPC NPs (see *Methods*). **(A–C)** Body weight change **(A)**, clinical disease score **(B)** and Kaplan-Meier survival curves **(C)** at the time points indicated on the *x* axis. *P < 0.05 (aAPC NPs vs controls). **(D, E)** Comparisons between mice receiving aAPC NPs (NPs) or not (ctr) for skin appearance **(D)** and tissue histopathology of lung, liver and colon (20 x magnification) **(E)**.

use. In general, the small number of Tregs that circulate in the peripheral blood requires Tregs expansion *ex vivo* before infusion in sufficient numbers. This associates with significant costs and specific technical requirements (25). Additionally, repeated treatments for the patient are often required, since *ex vivo*-expanded Tregs can become instable over time (26). Finally, chronic inflammation in autoimmune patients promotes the reversal of the phenotype of the transferred Tregs into T effector cells (27), and Treg potency may decrease over time (28).

Here we report that aAPC NPs can sustain Treg activity with prolonged efficacy in humanized mice, providing rational grounds for an immunotherapeutic expansion *in vivo* of human Tregs for the suppression of proinflammatory responses. Future investigations will address whether the inclusion of antigen-specificity in the aAPC NPs can further improve their tolerogenic benefits.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institutional Review Board of the University of California Los Angeles. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements. The animal study was reviewed and approved by the Animal Research Committee of the University of California Los Angeles.

AUTHOR CONTRIBUTIONS

ALC and DH designed the study. SG, SB, and ALC performed experiments. SG, DH, and ALC analyzed and discussed the results and gave critical intellectual contributions. ALC wrote the manuscript. DH contributed to its final editing. All authors contributed to the article and approved the submitted version.

FUNDING

Supported in part by the NIH grants HD097531 and AI154935 to ALC and the NIH grant GM007205 to SB.

ACKNOWLEDGMENTS

The authors acknowledge Tarek M. Fahmy's work in the field as stimulating the herein reported investigations.

REFERENCES

1. Turtle CJ, Riddell SR. Artificial Antigen-Presenting Cells for Use in Adoptive Immunotherapy. *Cancer J* (2010) 16:374–81. doi: 10.1097/PPO.0b013e3181eb33a6
2. Maus MV, Riley JL, Kwok WW, Nepom GT, June CH. HLA Tetramer-Based Artificial Antigen-Presenting Cells for Stimulation of $CD4^+$ T Cells. *Clin Immunol* (2003) 106:16–22. doi: 10.1016/S1521-6616(02)00017-7
3. Hasan AN, Selvakumar A, Doubrovina E, Riviere I, Sadelain MW, O'Reilly RJ. Artificial Antigen Presenting Cells That Express Prevalent HLA Alleles: A Step Towards the Broad Application of Antigen-Specific Adoptive Cell Therapies. *Discovery Med* (2009) 8:210–8.
4. Hasan AN, Selvakumar A, O'Reilly RJ. Artificial Antigen Presenting Cells: An Off the Shelf Approach for Generation of Desirable T-cell Populations for Broad Application of Adoptive Immunotherapy. *Adv Genet Eng* (2015) 4: 130. doi: 10.4172/2169-0111.1000130
5. Couture A, Garnier A, Docagne F, Boyer O, Vivien D, Le-Mauff B, et al. HLA-Class II Artificial Antigen Presenting Cells in $CD4^+$ T Cell-Based Immunotherapy. *Front Immunol* (2019) 10:1081. doi: 10.3389/fimmu.2019.01081
6. Horwitz DA, Bickerton S, Koss M, Fahmy TM, La Cava A. Suppression of Murine Lupus by $CD4^+$ and $CD8^+$ Treg Cells Induced by T Cell-Targeted Nanoparticles Loaded With Interleukin-2 and Transforming Growth Factor β. *Arthritis Rheumatol* (2019) 71:632–40. doi: 10.1002/art.40773
7. Horwitz DA, Fahmy TM, Piccirillo CA, La Cava A. Rebalancing Immune Homeostasis to Treat Autoimmune Diseases. *Trends Immunol* (2019) 40:888–908. doi: 10.1016/j.it.2019.08.003
8. Iikuni N, La Cava A. Murine models of lupus for the study of human SLE. In: A La Cava, editor. *Recent research developments in rheumatology*. Trivandrum: Research Signpost (2009). p. 55–78.
9. Naserian S, Leclerc M, Thiolat A, Pilon C, Le Bret C, Belkacemi Y, et al. Simple, Reproducible, and Efficient Clinical Grading System for Murine Models of Acute Graft-Versus-Host Disease. *Front Immunol* (2018) 9:10. doi: 10.3389/fimmu.2018.00010
10. Hahn BH, Anderson M, Le E, La Cava A. Anti-DNA Ig Peptides Promote Treg Cell Activity in Systemic Lupus Erythematosus Patients. *Arthritis Rheumatol* (2008) 58:2488–97. doi: 10.1002/art.23609
11. Nigam P, Velu V, Kannanganat S, Chennareddi L, Kwa S, Siddiqui M, et al. Expansion of $FOXP3^+CD8$ T Cells With Suppressive Potential in Colorectal Mucosa Following a Pathogenic Simian Immunodeficiency Virus Infection Correlates With Diminished Antiviral T Cell Response and Viral Control. *J Immunol* (2010) 184:1690–701. doi: 10.4049/jimmunol.0902955
12. Churlaud G, Pitoiset F, Jebbawi F, Lorenzon R, Bellier B, Rosenzwajg M, et al. Human and Mouse $CD8^+CD25^+FOXP3^+$ Regulatory T Cells At Steady State and During Interleukin-2 Therapy. *Front Immunol* (2015) 6:171. doi: 10.3389/fimmu.2015.00171
13. Bjarnadottir U, Lemarquis AL, Halldorsdottir S, Freysdottir J, Ludviksson BR. The Suppressive Function of Human $CD8^+$ iTregs is Inhibited by IL-1-β, and TNF-α. *Scand J Immunol* (2014) 80:313–22. doi: 10.1111/sji.12212
14. Pellegrino M, Crinò A, Rosado MM, Fierabracci A. Identification and Functional Characterization of $CD8^+$ T Regulatory Cells in Type 1 Diabetes Patients. *PloS One* (2019) 14:e0210839. doi: 10.1371/journal.pone.0210839
15. Dinesh RK, Skaggs BJ, La Cava A, Hahn BH, Singh RP. $CD8^+$ Tregs in Lupus, Autoimmunity, and Beyond. *Autoimmun Rev* (2010) 9:560–8. doi: 10.1016/j.autrev.2010.03.006
16. Hoffmann P, Eder R, Boeld TJ, Doser K, Piseshka B. Andreesen, R. Et Al. Only the $CD45RA^+$ Subpopulation of $CD4^+CD25^{high}$ T Cells Gives Rise to Homogeneous Regulatory T-cell Lines Upon *In Vitro* Expansion. *Blood* (2006) 108:4260–7. doi: 10.1182/blood-2006-06-027409
17. La Cava A. Natural Tregs and Autoimmunity. *Front Biosci* (2009) 14:333–43. doi: 10.2741/3247
18. Rhodes KR, Green JJ. Nanoscale Artificial Antigen Presenting Cells for Cancer Immunotherapy. *Mol Immunol* (2018) 98:13–8. doi: 10.1016/j.molimm.2018.02.016
19. Lü JM, Wang X, Marin-Muller C, Wang H, Lin PH, Yao Q, et al. Current Advances in Research and Clinical Applications of PLGA-based Nanotechnology. *Expert Rev Mol Diagn* (2009) 9:325–41. doi: 10.1586/erm.09.15
20. Clemente-Casares X, Blanco J, Ambalavanan P, Yamanouchi J, Singha S, Fandos C, et al. Expanding Antigen-Specific Regulatory Networks to Treat Autoimmunity. *Nature* (2016) 530:434–40. doi: 10.1038/nature16962
21. Serra P, Santamaria P. Nanoparticle-Based Autoimmune Disease Therapy. *Clin Immunol* (2015) 160:3–13. doi: 10.1016/j.clim.2015.02.003
22. Grant CR, Liberal R, Mieli-Vergani G, Vergani D, Longhi MS. Regulatory T-cells in Autoimmune Diseases: Challenges, Controversies and – Yet - Unanswered Questions. *Autoimmun Rev* (2015) 14:105–16. doi: 10.1016/j.autrev.2014.10.012
23. Giang S, La Cava A. Regulatory T Cells in SLE: Biology and Use in Treatment. *Curr Rheumatol Rep* (2016) 18:67. doi: 10.1007/s11926-016-0616-6
24. Horwitz DA, Pan S, Ou JN, Wang J, Chen M, Gray JD, et al. Therapeutic Polyclonal Human $CD8^+CD25^+Foxp3^+TNFR2^+PD\text{-}L1^+$ Regulatory Cells Induced *Ex-Vivo*. *Clin Immunol* (2013) 149:450–63. doi: 10.1016/j.clim.2013.08.007
25. Chakraborty R, Mahendravada A, Perna SK, Rooney CM, Heslop HE, Vera JF, et al. Robust and Cost Effective Expansion of Human Regulatory T Cells Highly Functional in a Xenograft Model of Graft-Versus-Host Disease. *Haematologica* (2013) 98:533–7. doi: 10.3324/haematol.2012.076430
26. Kim J, Hope CM, Perkins GB, Stead SO, Scaffidi JC, Kette FD, et al. Rapamycin and Abundant TCR Stimulation are Required for the Generation of Stable Human Induced Regulatory T Cells. *Clin Transl Immunol* (2020) 9:e1223. doi: 10.1002/cti2.1223
27. Wiesinger M, Stoica D, Roessner S, Lorenz C, Fischer A, Atreya R, et al. Good Manufacturing Practice-Compliant Production and Lot-Release of *Ex Vivo* Expanded Regulatory T Cells as Basis for Treatment of Patients With Autoimmune and Inflammatory Disorders. *Front Immunol* (2017) 8:1371. doi: 10.3389/fimmu.2017.01371
28. Yu Y, Liu Y, Shi F-D, Zou H, Hahn BH, La Cava A. Tolerance Induced by Anti-DNA Ig Peptide in $(NZB \times NZW)F_1$ Lupus Mice Impinges on the Resistance of Effector T Cells to Suppression by Regulatory T Cells. *Clin Immunol* (2012) 142:291–5. doi: 10.1016/j.clim.2011.11.004

Conflict of Interest: DH owns stocks and stock options at General Nanotherapeutics and Toralgen, Inc. DH has filed a US patent application (63118863) on the methods described in this manuscript to produce tolerogenic NPs for the treatment of immune-mediated diseases.

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.

frontiers in Immunology

REVIEW
published: 15 June 2021
doi: 10.3389/fimmu.2021.681062

Strategies to Use Nanoparticles to Generate CD4 and CD8 Regulatory T Cells for the Treatment of SLE and Other Autoimmune Diseases

David A. Horwitz[1,2]*, *Sean Bickerton*[3] *and Antonio La Cava*[4]

[1] General Nanotherapeutics, LLC, Santa Monica, CA, United States, [2] Department of Medicine, Keck School of Medicine, University of Southern California, Los Angeles, CA, United States, [3] Department of Biomedical Engineering, Yale University, New Haven, CT, United States, [4] Department of Medicine, University of California, Los Angeles, Los Angeles, CA, United States

Autoimmune diseases are disorders of immune regulation where the mechanisms responsible for self-tolerance break down and pathologic T cells overcome the protective effects of T regulatory cells (Tregs) that normally control them. The result can be the initiation of chronic inflammatory diseases. Systemic lupus erythematosus (SLE) and other autoimmune diseases are generally treated with pharmacologic or biological agents that have broad suppressive effects. These agents can halt disease progression, yet rarely cure while carrying serious adverse side effects. Recently, nanoparticles have been engineered to correct homeostatic regulatory defects and regenerate therapeutic antigen-specific Tregs. Some approaches have used nanoparticles targeted to antigen presenting cells to switch their support from pathogenic T cells to protective Tregs. Others have used nanoparticles targeted directly to T cells for the induction and expansion of CD4+ and CD8+ Tregs. Some of these T cell targeted nanoparticles have been formulated to act as tolerogenic artificial antigen presenting cells. This article discusses the properties of these various nanoparticle formulations and the strategies to use them in the treatment of autoimmune diseases. The restoration and maintenance of Treg predominance over effector cells should promote long-term autoimmune disease remission and ultimately prevent them in susceptible individuals.

Keywords: nanoparticles, regulatory T cells, systemic lupus erythematosus, autoimmunity, treatment, antigen-presenting cell, dendritic cell

OPEN ACCESS

Edited by:
Qizhi Tang,
University of California, San Francisco, United States

Reviewed by:
Masayuki Mizui,
Osaka University, Japan
Jamie Berta Spangler,
Johns Hopkins University, United States

***Correspondence:**
David A. Horwitz
dhorwitz@usc.edu

Specialty section:
This article was submitted to Immunological Tolerance and Regulation, a section of the journal Frontiers in Immunology

Received: *15 March 2021*
Accepted: *11 May 2021*
Published: *15 June 2021*

Citation:
Horwitz DA, Bickerton S and La Cava A (2021) Strategies to Use Nanoparticles to Generate CD4 and CD8 Regulatory T Cells for the Treatment of SLE and Other Autoimmune Diseases. Front. Immunol. 12:681062. doi: 10.3389/fimmu.2021.681062

INTRODUCTION

A major unmet need in chronic immune-mediated inflammatory diseases that include autoimmune diseases, graft versus host disease and allograft graft rejection is to achieve long-term remission. Most current approaches use agents that are only partially effective because they not only suppress pathologic cells but also the cells that are required to control those pathologic cells. Moreover, the broad immunosuppressive effects of pharmacological and/or biological agents are often accompanied by toxic side effects. Fortunately, novel strategies with more selective cellular targets (and thus more effective and less toxic) are being developed.

Autoimmune diseases are generally T cell-dependent disorders of the immune regulation. The immune system is constitutively highly active with a rapid turnover of T regulatory cells (Tregs) and antigen-presenting dendritic cells (DCs). Homeostatic regulatory mechanisms control immune cells with dual functions: 1) they fight infectious agents and 2) also prevent the emergence of potentially pathologic self-reactive cells not eliminated at birth. In health, regulatory populations of CD4+ and CD8+ Tregs keep these cells dormant, and interactions between tolerogenic DCs and Tregs maintain immune tolerance. In autoimmune diseases, instead, homeostasis becomes dysregulated and immunogenic DCs enable pathogenic T effector cells to predominate over the Tregs (1). A prototypical disorder of immune regulation is systemic lupus erythematosus (SLE), a multisystem autoimmune disease (2). In SLE both CD4+ and CD8+ Treg function is decreased (3).

Several therapeutic approaches have been developed to restore normal numbers and/or function of Tregs when abnormal. One approach that has reached clinical trials has been to isolate and expand the small numbers of Tregs present in the peripheral blood. The adoptive transfer of expanded autologous CD4 Tregs has been used to treat various autoimmune diseases, graft versus host disease and to prevent solid organ graft rejection (4). Adoptive CD4+ T cell therapy in one case of lupus with skin disease revealed evidence of T reg activation (5). Although the adoptive transfer of expanded polyclonal CD4 Tregs appears to be safe, the cost and technical complexity to expand autologous Tregs have limited this approach (6). An alternative strategy has been the induction/expansion of Tregs *ex vivo*. The cytokines interleukin (IL)-2 and transforming factor-beta (TGF-β) are essential for the generation, function and survival of CD4 Tregs (7, 8). In SLE, the production of IL-2 and TGF-β is decreased (9, 10). To treat SLE and other autoimmune diseases with low IL-2 production, one could induce and expand autologous SLE CD4 Tregs *ex vivo* with IL-2 and TGF-β for subsequent adoptive transfer of these cells back to the donor (11). Although this Treg-based therapeutic approach has been successful in mouse models, it has not yet reached the clinic. The possibility to induce and expand *in vivo* Tregs has recently been considered through the use of nanoparticles (NPs). Formulated NPs with the potential to reset the homeostatic mechanisms restoring Treg predominance are discussed here. Since DCs control T cell differentiation, one approach is to switch disease-associated immunogenic DCs to tolerogenic DCs (which induce and expand Tregs). Another approach directly targets T cells and increases functional CD4+ and CD8+ Tregs. We discuss how the immunotherapeutic use of NPs could lead to the reversal, long-term remission, and ultimately, prevention of autoimmune diseases.

NANOPARTICLES IN IMMUNOTHERAPY

Nanoparticles engineered to target specific cells or tissues with a high drug loading capacity represent a new generation of drug delivery systems for many biomedical indications. Nanoparticles are constructed using natural or synthetic materials with well-established safety record and have a typical diameter ranging from 0.1 to $1000x10^{-9}$ m (1 nanometer, which is 10x the size of an atom). The motivation for using such systems derive from the fact that viruses and pathogens that elicit or subvert immune responses are, in essence, small particles endowed with the ability to interact with - or avoid - immune cells in a variety of ways. Nanoparticles currently used consist of both organic or colloidal NPs that can be taken up by cells of the reticuloendothelial system. These include the phagocytic cells of the innate immune system such as macrophages, DCs and neutrophils. Other NPs can be surface-modified to target specific lymphocyte populations.

Advantages of NPs over traditional drugs include: 1) markedly decrease the amount of a biological agent delivered by 100 to 1000-fold when targeted to specific cells (by increasing the local concentration following release). This reduces the side effects as well as the cost. 2) improve the delivery of insoluble drugs and maximize bioavailability; 3) combine therapeutic agents with a diagnostic, resulting in "theranostic" agents. The durability of the concept is an indication of its appeal in developing immunomodulatory strategy technologies. The potential to assemble such materials on nanoscale dimension facilitates circulation in the blood, biodistribution to lymph nodes, interaction with extracellular receptors (if targeted appropriately) and intracellular accumulation without compromising normal physiologic functions. We focus here on the application of nanoparticles in the size range 100-500 nm (**Figure 1**).

Nanoparticles are currently being tested for the treatment of autoimmune disease because they can be engineered for three distinct uses: 1) they can function as carriers of biologic agents and small molecule drugs, 2) they can be anti-inflammatory, or 3) tolerogenic (12, 13). Taking advantage of the fact that they are phagocytosed by macrophages, NPs can encapsulate agents that polarize those cells to become anti-inflammatory. These agents include cytokines such as IL-10, statins, angiotensin receptor blockers, or peroxisome proliferator-activated receptor-γ (PPARγ) agonists (14). Nanoparticles loaded with biological agents such as tumor necrosis factor antagonists ameliorate inflammatory arthritis (15). Here we concentrate on the use of NPs to induce and expand Tregs.

The effects of NPs are determined by their size, biodistribution and route of administration. Particles smaller than 6 nm drain to the blood while particles larger than 9 nm drain preferentially to lymphatics. Particles 20 to 100 nm are taken up by liver sinusoidal cells or macrophages. Particles 100 to 200 nm traffic to the spleen and liver, and those up to 5 μm will accumulate in the spleen. NPs delivered by intravenous injection target APCs in the spleen and liver. Those delivered by subcutaneous injection are preferentially taken up by DCs in draining lymph nodes.

The materials used for the preparation of NPs can include metals, liposomes and synthetic and natural polymers (16–19). Specifically, polymers fabricated from polylactides (PLA) and copolymers of lactide and glycolide (poly-lactic-*co*-glycolic acid, PLGA) have established commercial use in humans and have a long safety record (20, 21). These systems have several features

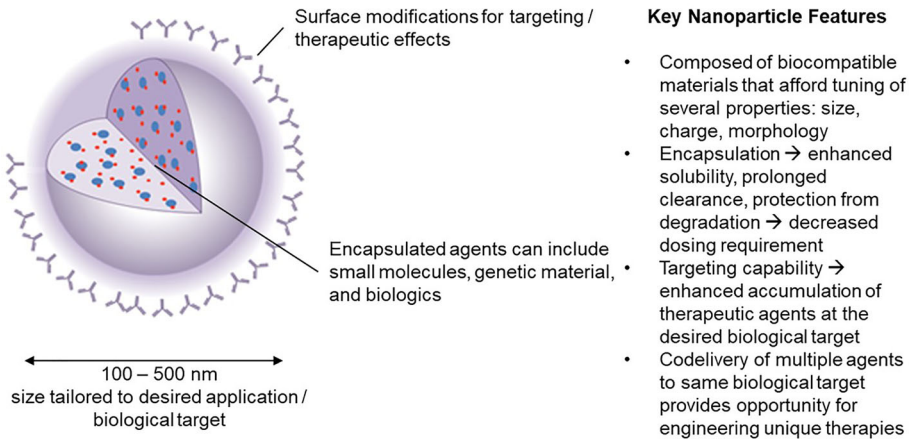


FIGURE 1 | Nanoparticle carriers offer a unique set of characteristics that have inspired significant interest in their use in engineering novel immunotherapies in the field of tolerance induction.

that make them ideal materials for the fabrication of anti-inflammatory or tolerogenic nanosystems: 1) control over the size range of fabrication, down to 100 nm and potentially even lower (an important feature for passing through biological barriers); 2) reproducible biodegradability without the addition of enzymes or cofactors; 3) capability for sustained release of encapsulated, protected cytokines or other agents that may be tuned in the range of days to months by varying factors such as the PLA to polymers of glycolic acid (PGA) copolymer ratios, potentially abrogating repetitive administrations, 4) well-understood fabrication methodologies that offer flexibility over the range of parameters that can be used for fabrication, including choices over the polymer material, solvent, stabilizer, and scale of production and 5) control over surface properties facilitating the introduction of targeting ligands on the surface (18, 22).

While other materials can be considered such as metal oxide NPs - which can be conjugated with antigens, targeting ligands and immunomodulators on the cell surface - these do not facilitate sustained release and are limited to applications that do require biodegradability. Renal clearance is the major clearance pathway with such systems and requires them to be ultra-small (<50 nm). Given the potential safety issues with long-term use, liposomes that carry antigen, NF-kB inhibitors, or immunosuppressive drugs are often safer options and do not require stringent size engineering criteria. They have been used to suppress arthritis and lupus (23, 24) and variants of liposomes with a hydrogel interior (to facilitate sustained release) have been developed and utilized for the delivery of biologics and small molecule drugs in lupus therapy (24).

The appeal of biodegradability of NPs for controlled release of encapsulant together with safety requirements have led to the wide use of synthetic biopolymers as materials for construction of biodegradable NPs. The most widely used NPs are synthetic polymers, such as PLA or PLGA. Unlike liposomes, which burst release unless lipids are cross-linked or the interior is modified with a hydrogel (12), these solid biodegradable polymer particles are stable over time in aqueous media, releasing encapsulant slowly and, in addition, they can be manufactured by a number of methodologies and facilitate encapsulation of hydrophobic moieties such as rapamycin, mycophenolic acid, vitamin D3 and dexamethasone (25) through an entanglement with the hydrophobic polymer core (24, 26–28). One group compared the tolerogenic effects of PLGA NPs with TMC-TPP (N-trimethyl chitosan tripolyphosphate) NPs. They found that PLGA NPs enhanced production of retinal dehydrogenase by APCs. This enzyme increases retinoic acid which enhanced CD4+Foxp3+ Tregs induced by TGF-β (29). Clinically, this is of interest because IL-2 and TGF-β induce human naïve CD4 cells to express FoxP3 but, unlike mice, these cells lack strong suppressive effects. Adding all-trans retinoic acid to IL-2 and TGF-β markedly increased the protective properties of the Tregs to levels equivalent to mouse Tregs (30). PLGA NPs also increase the stability of induced CD4 Tregs. As will be discussed below, mouse CD4+ cells induced to become CD25+Foxp3+ Tregs with IL-2- and TGF-β-loaded PLGA NPs were more stable than Tregs induced with soluble IL-2 and TGF-β (31).

RATIONALE FOR THE USE OF NANOPARTICLES

In the steady state, rapidly turning over immature DCs become tolerogenic and induce Tregs that maintain immune tolerance. In autoimmune diseases, instead, immature DCs become immunogenic and support pathogenic effector cells, with resulting predominant pathogenic T cells over the regulatory cells that should control them. The therapeutic objective, then, is to formulate NPs that can reset a dysregulated immune system back to normal and restore autoantigen specific Treg predominance. Since in some autoimmune diseases such as

SLE, type 1 diabetes (T1D) and multiple sclerosis, specific autoantigen peptides have been identified, the goal is to induce antigen-specific Tregs. However, in diseases such as rheumatoid arthritis and inflammatory bowel disease where specific autoantigens are unclear, the goal is to target NPs to disease sites to switch immunogenic DCs to tolerogenic DCs and switch local macrophages from inflammatory to anti-inflammatory cells.

To restore Treg predominance, two approaches are possible: 1) NPs targeted to DCs or other antigen-presenting cells (APCs), to induce them to become tolerogenic, or 2) NPs targeted directly to T cells for the induction and expansion of Tregs. **Figures 2** and **3** summarizes these approaches. It has been established that CD4 Tregs require IL-2, TGF-β, and continuous T cell receptor stimulation for function and survival (7, 32, 33). Nanoparticles can provide these agents and, where possible, the antigen for the generation of antigen-specific Tregs. Also, although most investigators have focused on CD4 Tregs, CD8 Tregs have as well important tolerogenic roles (34, 35). In human SLE, like CD4 Tregs, CD8 Tregs can inhibit anti-DNA autoantibodies (36, 37). Therefore, attention should be given to inducing CD8 as well as CD4 Tregs.

NANOPARTICLES THAT GENERATE TOLERANCE THROUGH MODULATION OF ANTIGEN PRESENTING CELLS (APCs)

Delivering Pharmacological Agents to Promote Tolerogenic APCs

The liver and the intestinal immune system are enriched in APCs with high tolerogenic potential (38, 39). It is well known that oral administration of protein antigens can result in non-responsiveness to those antigens. Oral tolerance can prevent certain autoimmune diseases in animals but, unfortunately,

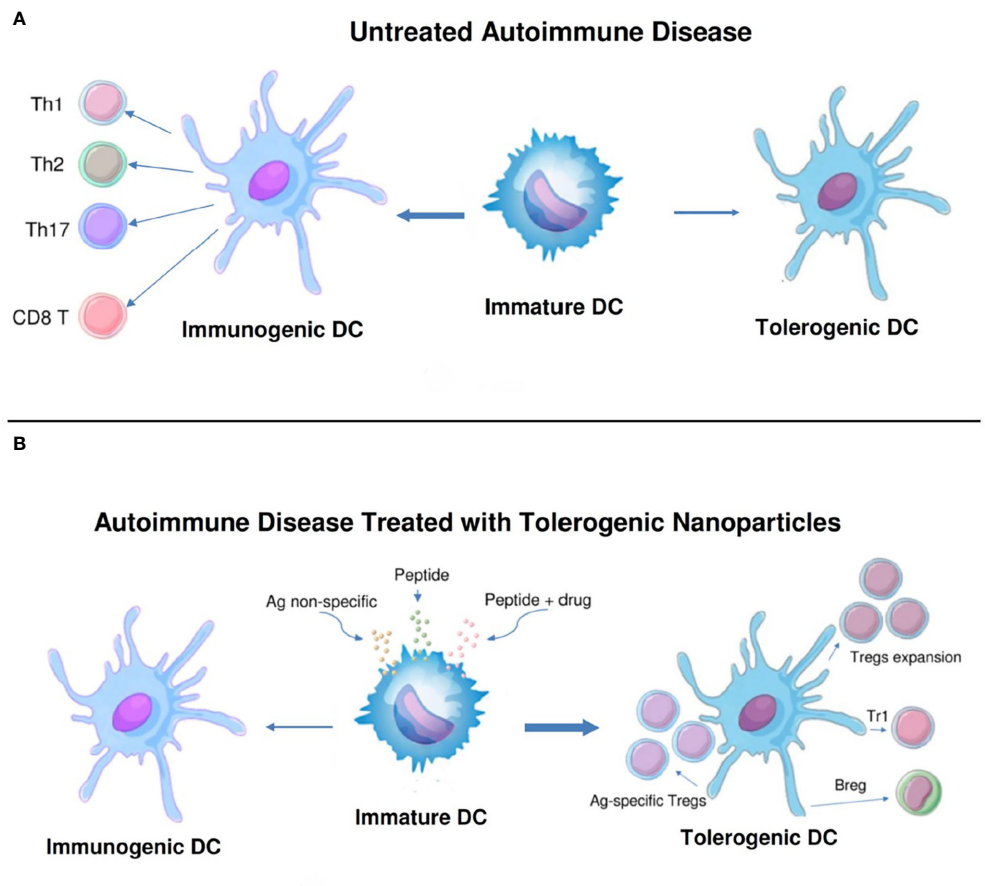


FIGURE 2 | Nanoparticles targeted to antigen-presenting cells can switch immunogenic dendritic cells to tolerogenic. **(A)**. While immature dendritic cells (DCs) normally mature to tolerogenic in the steady state, in untreated autoimmune disease these cells can become immunogenic and induce pathogenic T cell effector cells ($CD4^+$ Th1, Th2 and Th17, and $CD8^+$ T cells). **(B)**. Different formulations of nanoparticles (antigen non-specific, peptide-containing, or peptide plus drug) have been designed to switch the maturation of DCs from immunogenic back to tolerogenic. These DCs expand one or more populations of regulatory cells (antigen-specific and non-specific $CD4^+$ and $CD8^+$ Tregs, Tr1 cells, and B regulatory cells) and reset the immune system to restore a predominance of regulatory cells over pathogenic effector cells.

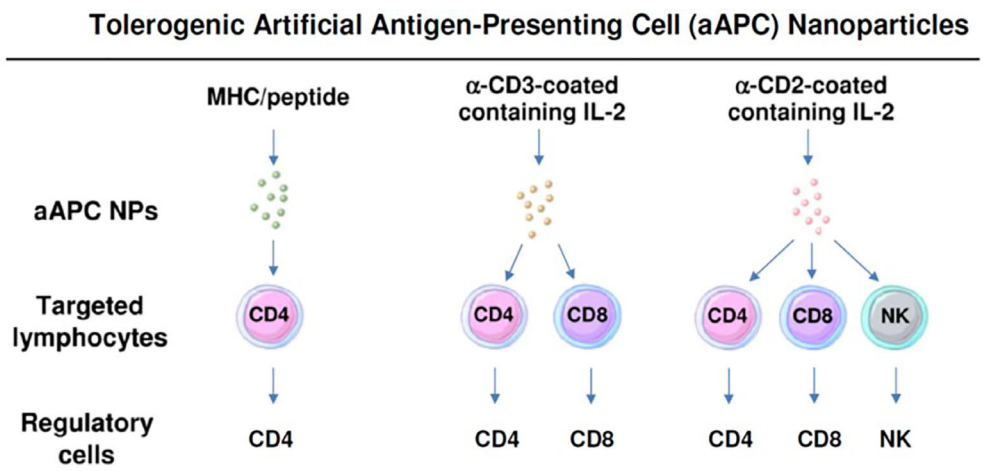


FIGURE 3 | Nanoparticles can be formulated as tolerogenic artificial antigen-presenting cells that directly target specific lymphocyte subpopulations to become regulatory cells. Three examples are shown that induce one or more subsets of regulatory cells.

multiple attempts to translate that to human therapeutics has not been successful (39). Nanoparticles have been used to amplify tolerogenic effects (40). Repeated oral delivery of chitosan-DNA NPs can prevent antibodies blocking functional FVIII in mice with hemophilia A (41). Oral gene application using chitosan-DNA NPs induce transferable tolerance (42). Orally delivered nanoparticle-curcumin has been reported to ameliorate experimental colitis *via* modulation of gut microbiota and the induction of Tregs (43, 44). Curcumin is a hydrophobic polyphenol prepared from the root of the perennial herb *Curcuma longa*, a member of the ginger family. Curcumin possesses a wide variety of biological functions, such as anti-inflammatory, anti-cancer, antioxidant, antimicrobial, wound-healing and hypoglycemic activities. Curcumin inhibits cell signaling pathways that include nuclear factor k light-chain-enhancer of activated B cells (NF-κB), signal transducer and activator of transcription proteins (STAT)3, nuclear factor erythroid 2-related factor 2 (Nrf2), reactive oxygen species (ROS), cyclooxygenase (COX)-2, and phosphatidylinositol 3-kinase (PI3K) (43). Strong cell signaling through NF-kB and the PI3K/Akt/mTOR pathway generates inflammatory cells or T effector cells, respectively, while weaker signals induce anti-inflammatory cells or Tregs. It is likely that modulating signaling from strong to weaker contribute to the many effects of curcumin (44). A recent breakthrough in the development of NPs capable of delivering biologicals orally will be described below.

Delivering Disease-Relevant Antigens to APCs Through Naturally Tolerogenic Mechanisms

In the steady state, a variety of APCs in the liver are in a tolerogenic state and maintain local and systemic immune tolerance to self and foreign antigens. These APCs include DCs, macrophage-like Kupffer cells (KCs), liver sinusoidal endothelial cells (LSECs), and hepatic stellate cells (HSCs). Even hepatocytes can express low levels of major histocompatibility complex (MHC)-I/MHC-II and co-stimulatory molecules that maintain tolerance (38). Investigators have taken advantage of NPs that accumulate in tolerogenic liver APCs to treat autoimmune diseases (45–47). One group has used PLGA NPs targeted to the liver to induce antigen-specific immune tolerance in a pulmonary allergen sensitization model (48). Others have used nanoparticle-based autoantigen delivery to Treg-inducing liver sinusoidal endothelial cells to control autoimmunity in mice (49).

Various approaches have been investigated that combine antigen delivery with a strong tolerogenic signal. The objective is to induce rapidly turning-over immature dendritic cells (DCs) to differentiate into tolerogenic APCs. In subjects with autoimmune diseases, immature DCs become immunogenic cells which perpetuate the disease. Here one must switch their differentiation of immunogenic cells to tolerogenic. One approach is to take advantage of the tolerogenic effects of clearing cells that died of apoptosis. Macrophages and immature APCs that phagocytose apoptotic cells produce TGF-β, which has tolerogenic effects (50–52). One group used the tolerogenic effects of apoptotic cells as a starting point for immunotherapy using the experimental allergic encephalomyelitis (EAE) mouse model of multiple sclerosis, characterized by T helper type 1 (Th1) and/or Th17 effector cells. The authors found that intravenous administration of peptides crosslinked to syngeneic splenic leukocytes safely and efficiently induced antigen-specific immune responses, and that the tolerance by apoptotic antigen-coupled leukocytes was induced by PD-L1+ and IL-10-producing splenic macrophages and maintained by Tregs (53). The same group then switched from antigen-labeled cells to antigen-bearing NPs. They observed that intravenous delivery of negatively charged PLGA NPs were taken up by splenic macrophages that express the scavenger receptor MARCO. These NPs prevented/treated EAE and T1D (54, 55), and the apoptotic effect of NPs carrying

antigen taken up by phagocytic immature APCs led to the production of and TGF-β and IL-10. These cytokines matured the APCs into tolerogenic, with the ability to induce Tregs. Most recently, it has been shown that PLGA NPs carrying single or multiple peptides could induce CD4+Foxp3+ Tregs that suppressed CD4 and CD8 cells (56).

Macrophage recognition of phosphatidylserine, a component of the cell membrane, is another strong apoptotic signal that can increase tolerogenic IL-10 and TGF-β. Liposomes containing peptide antigen and phosphatidylserine were given to patients with T1D to determine their tolerogenic effects on DCs. These liposomes decreased the autologous T cell proliferation. However, likely because of variability of the DC responses, liposomes did not affect the profile of pro-inflammatory or anti-inflammatory cytokines released by the cells (57).

Delivering Drug-Antigen Combinations to Drive Antigen Specific Tolerogenic Skewing

Nanoparticles that carry antigen-peptides and pharmacological agents have been studied for their capacity to generate Tregs. These agents attached to the surface or encapsulated in the PLGA NPs include TGF-β (58) and dexamethasone (59). Various immunomodulators have been used together with antigen-peptides to induce Tregs. Colloidal gold NPs have been engineered to deliver both a tolerogenic aryl hydrocarbon receptor (AHR) ligand and a proinsulin peptide to induce tolerogenic DCs that promote CD4+Foxp3+ Treg generation *in vivo* and prevent T1D in mice (60). These NPs induce monocyte-derived DCs to develop a tolerogenic phenotype by inhibiting NFkB signaling. The strength of cell signaling plays an important role in cell differentiation. The development of mature immunogenic DCs requires strong NFkB pathway signaling (61). By contrast, weaker NF-κB signaling is important in the establishment of immune tolerance, including both central tolerance and the peripheral function of Tregs (62). This AHR effect depends upon the induction of the suppressor of cell cytokine-2 (SOCS2) protein (60). These AHR-ligand containing NPs have been previously shown to induce Type 1 (Tr-1) Tregs and B regulatory cells (63). More recently this group has used nanoliposomes carrying an AHR ligand to treat EAE (64).

PLGA NPs containing antigen and an inhibitor of the PI3K/AKT/mTOR pathway have also been extensively studied for their tolerogenic effects. The PI3K pathway is the chief signaling pathway that T cells use to transmit antigen stimuli from the TCR to the nucleus (65). Similar to NFkB, strong TCR signals result in T effector cell differentiation, whereas weaker signals results in Treg differentiation (66). Rapamycin (rapa) inhibits signaling through mTOR. Although rapa has immunosuppressive effects, the combination of this agent and IL-2 promotes the induction of CD4+CD25+Foxp3+ cells (65). Rapa packaged in PLGA NPs has much stronger immunomodulatory properties than its soluble form (67). Nanoparticles containing antigen and rapamycin induce CD4+Foxp3+ Tregs and prevent EAE (67, 68). Many of the biological agents now in use for the treatment of human autoimmune diseases are antigen and can elicit antibodies that block their therapeutic effects. Tolerogenic polylactide NPs that block the production of these antibodies can have useful beneficial effects and are in clinical trials (69).

It is desirable to have antigen in the NP, yet antigen non-specific microparticles can also be useful. Blocking the positive co-stimulatory effects immunogenic DCs can be therapeutic. In T1D, three antisense oligonucleotides contained in microspheres were targeted to the primary transcripts of CD40, CD80 and CD86 co-stimulatory molecules. The result was attenuated T cell signaling that induced CD4+Foxp3+ Tregs which reversed hyperglycemia (70). In a lupus-like disease model resulting from a CD4 helper cell-driven chronic graft versus host disease, NPs induced CD4 and CD8 polyclonal Tregs that prevented the disease (31) Here the antigen source was non-self MHC peptides. Thus, with persistent endogenous antigen stimulation, polyclonal Tregs can have therapeutic effects.

NANOPARTICLES WITH DIRECT TOLEROGENIC EFFECTS ON LYMPHOCYTE SUBSETS

Delivering Small-Molecule Drugs or miRNA to T Cells

Nanoparticles can have direct effects on T cells and B cells. NPs have been used to correct decreased T cell production of IL-2 and increased production of IL-17 in SLE (2). Calcium/calmodulin protein kinase IV has a role in both abnormalities. KN93, a small molecule inhibitor of this kinase, was encapsulated in a nanolipogel that was targeted to CD4+ cells. Previously, this group had reported that the soluble form of this inhibitor increased CD4+ Foxp3+ Tregs (71). Here the NPs markedly reduced murine EAE and SLE (72). T cells were not depleted, but Th17 cells were effectively blocked. In SLE lupus prone mice, targeted delivery of a CaMK4 inhibitor to podocytes preserved their ultrastructure, prevented immune complex deposition and crescent formation, and suppressed proteinuria. In animals exposed to adriamycin, podocyte-specific delivery of a CaMK4 inhibitor prevented and reversed podocyte injury and renal disease (73).

Aberrant DNA demethylation in T cells leads to T cell abnormalities in SLE and correlates with disease activity (74). 5-azacytidine, (5-azaC) a DNA methyltransferase inhibitor can correct these abnormalities. However, generalized hypomethylation can have many adverse side effects. Therefore, 5-azaC was packaged in liposomes that were targeted to either CD4 or CD8 cells. Each of these liposomes markedly improved nephritis in a mouse model of lupus. The mechanism of action on each T cell subset was different. The CD4-targeted liposomes increased Foxp3 expression, expanded CD4 Treg numbers and enhanced function. The CD8-targeted liposomes enhanced cytotoxicity of these cells and restrained the expansion of pathogenic TCR-$\alpha\beta^{+}CD4^{-}CD8^{-}$ double-negative T cells. Importantly, systemic azaC delivery did not have these positive therapeutic effects (75). Thus, established disease could be reversed in a mouse model, underlining the importance of targeting NPs to specific cells.

In addition to T cells, liposome NPs have been used to target antigen directly to B cells. Antigenic liposomes displaying CD22 ligands induce antigen-specific B cell tolerance (76) and apoptosis (77).

Nanoparticles packaged with microRNA-125a (miR-125) have been reported to ameliorate a mouse model of lupus by restoring the balance between effector and Tregs. A miRNA is a small non-coding RNA molecule that functions in RNA silencing and post-transcriptional regulation of gene expression. miRNAs regulate approximately 90% of protein-coding genes (78). MiR-125 may have an important role in immune tolerance. One group reported that miR-125 is decreased in SLE patients (79). To repair this defect and increase stability of this RNA, miR-125 was packaged into ~150 nM NPs consisting of polyethylene glycol, PGLA, and poly (L-lysine). These NPs were endocytosed into activated T cells that became Tregs when cultured with TGF-β. Comparative *in vivo* studies in lupus mice with free miR-125 revealed that the NPs increased RNA concentration in the spleen and prevented splenomegaly and renal disease. This was accompanied by increased percentages of CD4 Tregs and decreased percentages of CD4 Th17 cells. Thus, in SLE, these NPs appear to have major effects on restoring normal immune regulation (80). However, miR-125 may have different properties in other diseases. In rheumatoid arthritis, levels of miR-125a are high and correlate with other inflammatory markers (81). In bacterial sepsis, high levels of this miRNA correlate with acute respiratory distress syndrome (82).

NANOPARTICLES THAT FUNCTION AS TOLEROGENIC ARTIFICIAL ANTIGEN-PRESENTING CELLS (aAPCs) THAT PROVIDE ACTIVATING, COSTIMULATORY, AND CYTOKINE SIGNALS

Several groups have tried to substitute DCs or other APCs with NPs to make artificial antigen presenting cells (aAPCs). While previously immunogenic aAPCS had been formulated to enhance immunization (83), two approaches were undertaken to generate tolerogenic aAPCs. One provided both CD4+ and CD8+ cells the T cell receptor stimulation and cytokines to become Tregs. The other used NPs to present peptide-MHC complexes directly to T cells to induce CD8+ and CD4+ Tregs.

In 2011, it was shown that PLGA NPs coated with anti-CD4 antibodies and loaded with Leukemia Inhibitory Factor (LIF) induced mouse CD4+ cells to become CD4+ Foxp3+ Tregs (84). These NPs blocked the ability of IL-6 to induce CD4+ cells to become pro-inflammatory IL-17-producing cells. NPs encapsulated with LIF have used as neuroprotective in multiple sclerosis to repair myelin *in vivo (*85, 86). This work was followed up in 2015 by loading CD4-targeted PLGA NPs with IL-2 and TGF-β, the cytokines that induce Foxp3 Tregs. These NPs induced mouse CD4+ cells to become Tregs that, unlike those induced with soluble IL-2 and TGF-β, were stable in the presence of IL-6. The percentage of nanoparticle-induced CD4 Tregs and their suppressive activity *in vitro* was much greater than those induced *ex vivo* by soluble IL-2 and TGF-β. Since CD4 Tregs need continuous IL-2 exposure to maintain Foxp3 expression (87), a single dose of NPs sustained Foxp3 expression for 10 days. By contrast, those CD4 cells stimulated with soluble IL-2 and TGF-β had completely lost Foxp3 expression by this time (13). At present, clinical trials are underway with low dose IL-2 to treat SLE. One has been completed: NCT 02084238. Ongoing trials include: NCT02955615, NCT03312335, NCT03451422, NCT03782636, and NCT02411253. While increases in Foxp3 quickly fall after each dose of IL-2, one might anticipate that NPs targeted to CD4+ cells that are loaded with this cytokine will sustain Foxp3 expression longer.

PLGA NPs targeted to both CD4 and CD8 cells and encapsulated with IL-2 and TGF-β have been used to prevent a lupus-like syndrome (chronic graft versus host disease) (31). In their studies with Tregs induced *ex-vivo*, this group had documented that the combination of CD4 and CD8 Tregs was more effective than CD4 Tregs alone in preventing this lupus-like syndrome (88). Their objective, therefore, was to expand CD4 and CD8 Tregs *in vivo*. To do so, they coated the NPs with both anti-CD2 and anti-CD4 antibodies. Anti-CD2 antibody was chosen since it had been reported that these antibodies can also target natural killer (NK) cells (89). This model was chosen because of its rapid read-out. It involves the transfer of mouse DBA/2 T cells into (C57BL/6 × DBA/2) F1 (BDF1) mice. Unlike most mouse strains, DBA/2 mice lack T cells that can kill CD8 cells and the ensuing graft versus host disease, therefore, is characterized by unopposed T cell help for antibody production. The result is a rapid onset of anti-DNA autoantibody production and a rapidly lethal immune complex-induced glomerulonephritis. In this model, the administration of these T cell and NK cell-targeted NPs containing IL-2 and TGF-β markedly suppressed disease.

In addition to mouse cells, tolerogenic aAPC NPs containing IL-2 and TGF-β have induced human CD4+ and CD8+ cells become Foxp3+ Tregs that were functional both *in vitro* and modulated systemic autoimmunity in humanized NOD/SCID immunodeficient mice. For the *in vitro* studies, the NPs were coated with anti-CD3 and anti-CD28 antibodies. For the *in vivo* studies, the NPs were anti-CD3 antibody-coated NPs containing IL-2 and TGF-β. After the transfer of human PBMC to the immunodeficient mice, treatment with aAPC NPs for three weeks resulted in increased CD4+ and CD8+ Foxp3+ cells that persisted until the termination of experiment. This was accompanied by increased survival of the human anti-mouse GVHD (90).

Another approach to use NPs as aAPCs is to present peptide-MHC complexes directly to T cells. In 2010 one group used NPs that carried peptide-MHC class I complexes to delete a subset of diabetogenic CD8+ cells in NOD mice. Although these NPs did restore blood sugar to normal levels in mice with new-onset diabetes, they unexpectedly expanded a subset of CD8+ cells that were autoregulatory cytotoxic cells that suppressed polyclonal autoimmune responses by killing autoantigen-loaded APCs in target tissue and draining lymph nodes (91). These workers then

turned their attention to disease-relevant peptide-MHC class II complexes to expand therapeutic CD4 Tregs. They identified pMHC complexes that reversed diabetes, EAE and collagen arthritis in mice (92). The NPs targeted antigen-experienced pathogenic IFN-γ producing T helper 1 (Th1) cells and switched these cells into T regulatory type 1-like (Tr1-like) cells that produce predominantly anti-inflammatory IL-10. The Tr1 cells induced B cells to become IL-10-producing B regulatory cells. They documented ten pMHC class II complexes that had similar effects. Subsequently, they have identified complexes of non-organ peptides from mitochondria, nuclear or cytoplasmic proteins with MHCII have that induced therapeutic Tr1 cells in mouse models of liver diseases. These included primary biliary cirrhosis, primary sclerosing cholangitis and autoimmune hepatitis (93).

Another group engineered tolerogenic NPs co-coupling a myelin peptide-MHC complex, anti-Fas antibody, PD-L1-Fc and encapsulated with TGF-β These NPs decreased Th1, Th17, and Tc17 cells and increased Tregs. In EAE, mice that were treated early after disease onset responded well, but those treated with more advanced disease did less well (94). In addition to EAE, a study in skin transplantation with similar NPs co-coupling MHC class I dimers, CD47 and regulatory molecules showed that the NPs bound and induced apoptosis of CD8 cells, induced Tregs and improved transplant survival (95). Like the aAPC study described above, the work was conducted on mice with a C57/BL background. Since human autoimmune diseases occur in subjects with a much more diverse genetic background, obstacles remain for clinical translation as well as for manufacturing challenges.

NANOPARTICLES THAT INDUCE TOLEROGENIC TGF-β-DEPENDENT REGULATORY NK CELLS

Nanoparticles coated with anti-CD2 antibodies target NK cells as well as T cells. Studies were, therefore, undertaken to determine whether NK cells had a role in the protective effects of anti-CD2 antibody-coated NPs loaded with IL-2 and TGF-β in the lupus-like disease discussed above (96). Surprisingly, depletion of NK cells attenuated the NP-mediated increase in CD4+ and CD8+ Foxp3+ Tregs and exacerbated the resulting renal disease above the baseline of untreated mice (96).

Previously, anti-CD2 antibodies had been reported to induce NK cells to produce TGF-β (10, 97). This finding raised the possibility that TGF-β produced by NK cells could eliminate the need for this cytokine encapsulated in the anti-CD2 antibody-coated NPs. Additional studies were conducted with anti-CD2 antibody-coated NPs loaded with only IL-2 revealed that these NPs had equivalent protective effects on the renal disease as NPs containing both IL-2 and TGF-β. However, antagonizing TGF-β in the NP-treated mice by anti-TGF-β antibodies or with an Alk5 TGF-β signaling inhibitor abolished the protective effects. Thus, the protective effects of NPs loaded with only IL-2 were TGF-β-dependent.

Interestingly, NK cells harvested from the spleens of anti-CD2 antibody-coated NPs treated mice had equivalent protective effects on the lupus-like glomerulonephritis as the anti-CD2 antibody-coated NPs loaded with IL-2. Moreover, transfecting these NK cells with a silent RNA (sRNA) to inhibit TGF-β production completely abolished their protective effects. These studies provide evidence that the TGF-β produced by the NK cells may help in the maintenance and function of the CD4 and CD8 Tregs and, therefore, may play a major role in their protective effects (96).

NANOPARTICLES DELIVERED ORALLY WITH INHERENT ANTI-INFLAMMATORY AND TOLEROGENIC PROPERTIES

Orally delivered NPs have been used to treat T1D in nonobese diabetic (NOD) mice. Oral polyethylene glycol (PEG)-PLGA loaded with insulin lowered glucose in T1D rodent models (98, 99). Orally delivered PLGA NPs with al-trans retinoic acid and TGF-β induced therapeutic Tregs in T1D (increased PD-1 and CTLA4 but not Foxp3) (100). However, the oral bioavailability of these NPs is only 1-2% because of intestinal degradation (101).

Recently, it has been reported that NP polymerization of ursodeoxycholic acid (pUDCA), a bile acid with well-known anti-inflammatory and immunomodulatory effects, markedly enhanced its therapeutic properties. In addition, pUDCA NPs had the capability to deliver insulin orally without intestinal degradation. These NPs were rapidly absorbed intact and taken up by monocytes and intestinal macrophages that highly express bile acid TGR5 receptors. This interaction results in their differentiation to M2 anti-inflammatory macrophages, an effect which had important therapeutic consequences. Two different mouse models of Type 1 diabetes were successfully treated with pUDCA NPs. Cyclophosphamide-induced diabetes was prevented with pUDCA NPs containing rapamycin. Treatment of hyperglycemic NOD mice with PUDCA NPs containing insulin lowered blood glucose, reversed inflammation, and increased survival. In both models the ratio of cytotoxic CD8 cells and CD4 Tregs in draining lymph nodes was reversed, a finding suggesting that immunogenic dendritic cells had been switched to tolerogenic. Thus, pUDCA NPs appear to be a first in class orally ingestible carrier with remarkable therapeutic properties applicable to a wide variety of immune-mediated inflammatory diseases (102).

DISCUSSION AND CONCLUDING REMARKS

We have reviewed various approaches that use NPs to generate and expand Tregs by targeting APCs or directly targeting T cells and these approaches are summarized in **Table 1**. To induce and expand therapeutic polyclonal Tregs, NPs can be targeted to the large numbers of tolerogenic APCs present in the liver and in the

TABLE 1 | Different approaches employing nanoparticles therapies for tolerance induction.

Tolerogenic action through modulation of antigen-presenting cells

Category	NP Description	Mechanism	References
Delivery of pharmacological agents to promote tolerogenic APCs	Multiple polymer- (PLGA) or lipid-based (liposome) NP formulations encapsulating immunomodulatory agents such as rapamycin, dexamethasone, vitamin D3 and curcumin	Induction of tolerogenic dendritic cell phenotype that can promote tolerance through a variety of mechanisms including Treg expansion and anti-inflammatory cytokine production. No antigen-specificity	40, 43, 44, 70
Delivery of disease-relevant antigen to APCs through naturally tolerogenic mechanisms	PLGA or chitosan NPs with encapsulated antigen	Oral delivery → Oral Tolerance	41, 42, 98–101
	Antigen-loaded pUDCA NPs (additional immunosuppressive property of polymer material)		102
	Antigen-loaded NPs designed to display signatures of apoptotic cells to exterior; examples include surface-bound phosphatidylserine and negative surface charge to promote internalization by MARCO receptor	Mimicry of apoptotic cells/bodies	53–57
	Antigen-loaded PLGA coated with ligands for mannose/scavenger receptors on LSEC	Targeting of naturally tolerogenic environments (liver sinusoidal endothelial cells, LSEC)	48
	Polymer-coated iron oxide nanocrystals or quantum dots with conjugated peptide antigen		49
Delivery of drug-antigen combination to APCs	PLGA NPs with co-encapsulated rapamycin and antigen or rapamycin only (delivered with free antigen)	Antigen delivery to APCs which are skewed tolerogenic by codelivery of immunomodulatory agents.	15, 67–69
	Gold NPs with conjugated peptide antigen and tolerogenic aryl hydrocarbon receptor agonist (later work with liposomes)		60, 63, 64

Direct tolerogenic action on lymphocyte subsets

Category	NP Description	Mechanism	References
Delivery of small molecules to T cells	Nanolipogel system encapsulating CaMK4 inhibitor, KN93	Selective inhibition of CaMK4 in targeted CD4 T cells blocks Th17 differentiation	72, 73
	Nanolipogel system encapsulating DNA methyltransferase inhibitor, 5-aqzacytidine	Targeted demethylation leads to expansion and enhanced function of Tregs (CD4) cells and restrains expansion of pathogenic double-negative T cells (CD8)	75
Delivery of miRNA to T cells	Pegylated PLGA-b-poly(l-lysine) NP encapsulating miR-125a	Corrects imbalance of effector/regulatory T cells present in model of SLE	80
Delivery of cytokines to T cells	PLGA NPs encapsulating Leukemia Inhibitory Factor	Targeted delivery to CD4 T cells blocks IL-6 induced Th17 differentiation and favors upregulation of Tregs	84–86
	CD4/8-targeted PLGA NPs encapsulating TGF-β and IL-2	Paracrine delivery of cytokines promotes the induction and sustained expansion of CD4/8 Tregs with stable Foxp3 expression	13, 31, 90
	CD2-targeted PLGA NPs encapsulating TGF-β and IL-2	Targeted delivery of IL-2 to NK cells *via* anti-CD2 promotes expansion and upregulation of native TGF-β production	96
Peptide-MHC presentation to T cell receptors	pMHC complexes bound to surface of metal-oxide NPs	pMHC signal in the absence of costimulation promotes differentiation of IL-10 producing Tr1 cells and triggers deletion of pathogenic effector populations	91–93
Antigen delivery to B cells	Liposomes displaying both antigen and glycan ligands of CD22	Antigen exposure in the presence of CD22 engagement initiates tolerogenic programming that promotes antigen specific B cell tolerance as measured by decreased autoantibody formation	76, 77
Combination of multiple approaches	PLGA NPs decorated with pMHC, CD47, and multiple regulatory molecules with encapsulated TGFβ	Inhibition of T cell proliferation with selective decreases in effector Th1/Th17. Upregulation of regulatory T cells. Increased TGF-β and IL-10 in CNS and spleen.	94, 95

intestinal immune system. Antigen-specific Tregs can be induced by including peptide antigens carried by the NPs. In autoimmune diseases approaches are directed to switch the differentiation of rapidly turning-over immature dendritic cells from immunogenic to tolerogenic. These include peptide-loaded NPs formulated to mimic the tolerogenic effects of particle apoptosis. A pharmacologic agent can be attached to or encapsulated in these NPs to enhance their tolerogenic properties. Alternatively, tolerogenic NPs can be formulated that directly target T cells or NK cells. NPs coating with peptide/MHC complexes target Th1 T cells and can switch them to become Treg1 cells in MHC compatible subjects. NPs coated with anti-CD2 or anti-CD3 antibodies can act as artificial APCs that target CD4 and CD8 cells that provide the T cell receptor stimulation, IL-2 and TGF-β that induce and/or expand polyclonal Tregs. These NPs have the potential to repair defects in IL-2 and/or TGF-β production associated with SLE and other autoimmune diseases and, thus, normalize Treg function. Since these anti-CD2 and anti-CD3 antibody-coated NPs have the additional property to induce their targeted lymphocytes to provide TGF-β in the local environment. These NPs therefore, contain only IL-2 (96). Because of the pleotropic activities of

TGF-β, the possible adverse side effects of NPs containing TGF-β can be avoided. As indicated above, coating the NPs with anti-CD2 antibodies has recently been reported to induce NK cells to produce the TGF-β needed for the maintenance of Tregs.

There are significant challenges to be confronted in developing NN-based therapies for autoimmune diseases. First, the translation of laboratory formulations of therapeutic NPs up to large scale clinical grade numbers will be formidable (103). There are manufacturing challenges in standardization and quality control of large batches of NPs. Secondly, not only autoimmune diseases diverse in type, but the individual presentation of a given disease can vary considerably. The therapeutic effects can vary between the initial time of onset and the chronic phase of the disease. We believe the optimal time to treat these diseases will be early before organ damage occurs. We are also optimistic that NP treatment of highly susceptible subjects before the onset of clinical disease may be beneficial. For example, treatment of rheumatoid arthritis early with tumor necrosis factor antagonists had the best likelihood of achieving remission (104). Thirdly, the dose, timing and frequency of administration of the therapeutic NP must be carefully evaluated. Fourthly, in achieving the objective to induce antigen-specific Tregs, the causal peptide can differ in that patients affected. Finally, in clinical trials the concurrent use of other immunosuppressive drugs can greatly influence the therapeutic outcome.

Clinical trials using tolerogenic nanoparticle formulations have begun. The first indication has been to prevent the emergence of antibodies to biological agents that can interfere with their beneficial effects. Human proof-of concept for the mitigation of anti-drug antibodies has been demonstrated in a phase II study in patients with refractory gout with NPs that are that loaded with pegadricase, a pegylated formulation of uricase, an enzyme that breaks down uric acid. Since pegadricase is strongly immunogenic, the NPs also contain rapamycin which converts strong immunogenic signals mediated by the PI3K/Akt/mTOR pathway to weaker tolerogenic signals (69). In addition, clinical trials using low dose IL-2 to repair and enhance Treg function are in progress for the treatment SLE and other autoimmune diseases. In one of these studies patients with SLE and other chronic immune-mediated diseases were treated with intermittent doses of low dose IL-2 for 6 months with persistent increases in CD4 Tregs and clinical improvement of disease activity and severity (105).

Although the results with low dose IL-2 have been encouraging, it is likely that NPs directly targeted to T cells which are able to provide them the stimulation and small amounts of both IL-2 and TGF-β in the local environment for them to become Tregs can have even more beneficial therapeutic effects with additional safety. The judicious use of these NPs can possibly achieve long-term remission and, ultimately, prevent SLE and other chronic immune-mediated inflammatory diseases in highly susceptible individuals.

AUTHOR CONTRIBUTIONS

DAH wrote the manuscript. ALC and SB edited the manuscript, made direct intellectual contributions, and prepared the Table and Figures. All authors contributed to the work and approved it for publication.

FUNDING

Supported in part by the NIH grants HD097531 and AI154935 to ALC, and GM007205 to SB.

ACKNOWLEDGMENTS

The authors are grateful to Tarek M Fahmy for providing the inspiration and intellectual expertise that was helpful in gaining the background needed for the preparation of the manuscript.

REFERENCES

1. Horwitz DA, Fahmy TM, Piccirillo CA, La Cava A. Rebalancing Immune Homeostasis to Treat Autoimmune Diseases. *Trends Immunol* (2019) 40:888–908. doi: 10.1016/j.it.2019.08.003
2. Moulton VR, Suarez-Fueyo A, Meidan E, Li H, Mizui M, Tsokos GC. Pathogenesis of Human Systemic Lupus Erythematosus: A Cellular Perspective. *Trends Mol Med* (2017) 23:615–35. doi: 10.1016/j.molmed.2017.05.006
3. Horwitz DA. Regulatory T Cells in Systemic Lupus Erythematosus: Past, Present and Future. *Arthritis Res Ther* (2008) 10:227. doi: 10.1186/ar2511
4. Esensten JH, Muller YD, Bluestone JA, Tang Q. Regulatory T-Cell Therapy for Autoimmune and Autoinflammatory Diseases: The Next Frontier. *J Allergy Clin Immunol* (2018) 142:1710–8. doi: 10.1016/j.jaci.2018.10.015
5. Dall'Era M, Pauli ML, Remedios K, Taravati K, Sandova PM, Putnam AL, et al. Adoptive Treg Cell Therapy in a Patient With Systemic Lupus Erythematosus. *Arthritis Rheumatol* (2019) 71:431–40. doi: 10.1002/art.40737
6. Hippen KL, Riley JL, June CH, Blazar BR. Clinical Perspectives for Regulatory T Cells in Transplantation Tolerance. *Semin Immunol* (2011) 23:462–8. doi: 10.1016/j.smim.2011.07.008
7. Fontenot JD, Rasmussen JP, Gavin MA, Rudensky AY. A Function for Interleukin 2 in Foxp3-Expressing Regulatory T Cells. *Nat Immunol* (2005) 6:1142–51. doi: 10.1038/ni1263
8. Li MO, Sanjabi S, Flavell RA. Transforming Growth Factor-Beta Controls Development, Homeostasis, and Tolerance of T Cells by Regulatory T Cell-Dependent and -Independent Mechanisms. *Immunity* (2006) 25:455–71. doi: 10.1016/j.immuni.2006.07.011
9. Linker-Israeli M, Bakke AC, Kitridou RC, Gendler S, Gillis S, Horwitz DA. Defective Production of Interleukin 1 and Interleukin 2 in Patients With Systemic Lupus Erythematosus (SLE). *J Immunol* (1983) 130:2651–5.
10. Ohtsuka K, Gray JD, Stimmler MM, Toro B, Horwitz DA. Decreased Production of TGF-Beta by Lymphocytes From Patients With Systemic Lupus Erythematosus. *J Immunol* (1998) 160:2539–45.
11. Horwitz DA, Zheng SG, Gray JD, Wang JH, Ohtsuka K, Yamagiwa S. Regulatory T Cells Generated *Ex Vivo* as an Approach for the Therapy of Autoimmune Disease. *Semin Immunol* (2004) 16:135–43. doi: 10.1016/j.smim.2003.12.009
12. Look M, Stern E, Wang QA, DiPlacido LD, Kashgarian M, Craft J, et al. Nanogel-Based Delivery of Mycophenolic Acid Ameliorates Systemic Lupus Erythematosus in Mice. *J Clin Invest* (2013) 123:1741–9. doi: 10.1172/JCI65907

13. McHugh MD, Park J, Uhrich R, Gao W, Horwitz DA, Fahmy TM. Paracrine Co-Delivery of TGF-Beta and IL-2 Using CD4-Targeted Nanoparticles for Induction and Maintenance of Regulatory T Cells. *Biomaterials* (2015) 59:172–81. doi: 10.1016/j.biomaterials.2015.04.003
14. Di Mascolo D, Aryal S, Ramirez MR, Wang J, Candeloro P, Guindani M, et al. Rosiglitazone-Loaded Nanospheres for Modulating Macrophage-Specific Inflammation in Obesity. *J Controlled Release Off J Controlled Release Soc* (2013) 170:460–8. doi: 10.1016/j.jconrel.2013.06.012
15. Kishimoto TK, Ferrari JD, LaMothe RA, Kolte PN, Griset AP, O'Neil C, et al. Improving the Efficacy and Safety of Biologic Drugs With Tolerogenic Nanoparticles. *Nat Nanotechnol* (2016) 11:890–9. doi: 10.1038/nnano.2016.135
16. Metcalfe SM, Fahmy TM. Targeted Nanotherapy for Induction of Therapeutic Immune Responses. *Trends Mol Med* (2012) 18:72–80. doi: 10.1016/j.molmed.2011.11.002
17. Kishimoto TK, Maldonado RA. Nanoparticles for the Induction of Antigen-Specific Immunological Tolerance. *Front Immunol* (2018) 9:230. doi: 10.3389/fimmu.2018.00230
18. Look M, Bandyopadhyay A, Blum JS, Fahmy TM. Application of Nanotechnologies for Improved Immune Response Against Infectious Diseases in the Developing World. *Adv Drug Deliv Rev* (2010) 62:378–93. doi: 10.1016/j.addr.2009.11.011
19. Makadia HK, Siegel SJ. Poly Lactic-co-Glycolic Acid (PLGA) as Biodegradable Controlled Drug Delivery Carrier. *Polym (Basel)* (2011) 3:1377–97. doi: 10.3390/polym3031377
20. Jiang S, Camara N, Lombardi G, Lechler RI. Induction of Allopeptide-Specific Human CD4+CD25+ Regulatory T Cells *Ex Vivo*. *Blood* (2003) 102:2180–6. doi: 10.1182/blood-2003-04-1164
21. Bramwell VW, Eyles JE, Oya Alpar H. Particulate Delivery Systems for Biodefense Subunit Vaccines. *Adv Drug Deliv Rev* (2005) 57:1247–65. doi: 10.1016/j.addr.2005.01.010
22. Park J, Mattessich T, Jay SM, Agawu A, Saltzman WM, Fahmy TM. Enhancement of Surface Ligand Display on PLGA Nanoparticles With Amphiphilic Ligand Conjugates. *J Controlled Release Off J Controlled Release Soc* (2011) 156:109–15. doi: 10.1016/j.jconrel.2011.06.025
23. Capini C, Jaturanpinyo M, Chang HI, Mutalik S, McNally A, Street S, et al. Antigen-Specific Suppression of Inflammatory Arthritis Using Liposomes. *J Immunol* (2009) 182:3556–65. doi: 10.4049/jimmunol.0802972
24. Look M, Saltzman WM, Craft J, Fahmy TM. The Nanomaterial-Dependent Modulation of Dendritic Cells and Its Potential Influence on Therapeutic Immunosuppression in Lupus. *Biomaterials* (2014) 35:1089–95. doi: 10.1016/j.biomaterials.2013.10.046
25. Ilyinskii PO, Kovalev GI, O'Neil CP, Roy CJ, Michaud AM, Drefs NM, et al. Synthetic Vaccine Particles for Durable Cytolytic T Lymphocyte Responses and Anti-Tumor Immunotherapy. *PLoS One* (2018) 13:e0197694. doi: 10.1371/journal.pone.0197694
26. Almeria B, Deng W, Fahmy TM, Gomez A. Controlling the Morphology of Electrospray-Generated PLGA Microparticles for Drug Delivery. *J Colloid Interface Sci* (2010) 343:125–33. doi: 10.1016/j.jcis.2009.10.002
27. Almeria B, Fahmy TM, Gomez A. A Multiplexed Electrospray Process for Single-Step Synthesis of Stabilized Polymer Particles for Drug Delivery. *J Controlled Release Off J Controlled Release Soc* (2011) 154:203–10. doi: 10.1016/j.jconrel.2011.05.018
28. Demento SL, Cui W, Criscione JM, Stern E, Tulipan J, Kaech SM, et al. Role of Sustained Antigen Release From Nanoparticle Vaccines in Shaping the T Cell Memory Phenotype. *Biomaterials* (2012) 33:4957–64. doi: 10.1016/j.biomaterials.2012.03.041
29. Keijzer C, Spiering R, Silva AL, van Eden W, Jiskoot W, Vervelde L, et al. PLGA Nanoparticles Enhance the Expression of Retinaldehyde Dehydrogenase Enzymes in Dendritic Cells and Induce FoxP3(+) T-Cells In Vitro. *J Controlled release Off J Controlled Release Soc* (2013) 168:35–40. doi: 10.1016/j.jconrel.2013.02.027
30. Lu L, Zhou X, Wang J, Zheng SG, Horwitz DA. Characterization of Protective Human CD4CD25 FOXP3 Regulatory T Cells Generated With IL-2, TGF-Beta and Retinoic Acid. *PloS One* (2010) 5:e15150. doi: 10.1371/journal.pone.0015150
31. Horwitz DA, Bickerton S, Koss M, Fahmy TM, La Cava A. Suppression of Murine Lupus by CD4+ and CD8+ Treg Cells Induced by T Cell-Targeted Nanoparticles Loaded With Interleukin-2 and Transforming Growth Factor Beta. *Arthritis Rheumatol* (2019) 71:632–40. doi: 10.1002/art.40773
32. Li MO, Wan YY, Flavell RA. T Cell-Produced Transforming Growth Factor-Beta1 Controls T Cell Tolerance and Regulates Th1- and Th17-Cell Differentiation. *Immunity* (2007) 26:579–91. doi: 10.1016/j.immuni.2007.03.014
33. Levine AG, Arvey A, Jin W, Rudensky AY. Continuous Requirement for the TCR in Regulatory T Cell Function. *Nat Immunol* (2014) 15:1070–8. doi: 10.1038/ni.3004
34. Flippe L, Bezie S, Anegon I, Guillonneau C. Future Prospects for CD8+ Regulatory T Cells in Immune Tolerance. *Immunol Rev* (2019) 292:209–24. doi: 10.1111/imr.12812
35. Shimokawa C, Kato T, Takeuchi T, Ohshima N, Furuki T, Ohtsu Y, et al. CD8+ Regulatory T Cells Are Critical in Prevention of Autoimmune-Mediated Diabetes. *Nat Commun* (2020) 11:1922. doi: 10.1038/s41467-020-15857-x
36. Zhang L, Bertucci AM, Ramsey-Goldman R, Burt RK, Datta SK. Regulatory T Cell (Treg) Subsets Return in Patients With Refractory Lupus Following Stem Cell Transplantation, and TGF-Beta-Producing CD8+ Treg Cells are Associated With Immunological Remission of Lupus. *J Immunol* (2009) 183:6346–58. doi: 10.4049/jimmunol.0901773
37. Iikuni N, Lourenco EV, Hahn BH, La Cava A. Cutting Edge: Regulatory T Cells Directly Suppress B Cells in Systemic Lupus Erythematosus. *J Immunol* (2009) 183:1518–22. doi: 10.4049/jimmunol.0901163
38. Horst AK, Neumann K, Diehl L, Tiegs G. Modulation of Liver Tolerance by Conventional and Nonconventional Antigen-Presenting Cells and Regulatory Immune Cells. *Cell Mol Immunol* (2016) 13:277–92. doi: 10.1038/cmi.2015.112
39. Weiner HL. Oral Tolerance: Immune Mechanisms and the Generation of Th3-Type TGF-Beta-Secreting Regulatory Cells. *Microbes Infect* (2001) 3:947–54. doi: 10.1016/S1286-4579(01)01456-3
40. Garcia-Gonzalez P, Ubilla-Olguin G, Catalan D, Schinnerling K, Aguillon JC. Tolerogenic Dendritic Cells for Reprogramming of Lymphocyte Responses in Autoimmune Diseases. *Autoimmun Rev* (2016) 15:1071–80. doi: 10.1016/j.autrev.2016.07.032
41. Dhadwar SS, Kiernan J, Wen J, Hortelano G. Repeated Oral Administration of Chitosan/DNA Nanoparticles Delivers Functional FVIII With the Absence of Antibodies in Hemophilia A Mice. *J Thromb Haemost* (2010) 8:2743–50. doi: 10.1111/j.1538-7836.2010.04116.x
42. Goldmann K, Ensminger SM, Spriewald BM. Oral Gene Application Using Chitosan-DNA Nanoparticles Induces Transferable Tolerance. *Clin Vaccine Immunol* (2012) 19:1758–64. doi: 10.1128/CVI.00186-12
43. Qiao Q, Jiang Y, Li G. Inhibition of the PI3K/AKT-NF-kappaB Pathway With Curcumin Enhanced Radiation-Induced Apoptosis in Human Burkitt's Lymphoma. *J Pharmacol Sci* (2013) 121:247–56. doi: 10.1254/jphs.12149FP
44. La Cava A, Fiala M. Curcumin and Immunity. *Curr Bioact Compd* (2010) 6:156–160 6. doi: 10.2174/157340710793237335
45. Saito E, Kuo R, Kramer KR, Gohel N, Giles DA, Moore BB, et al. Design of Biodegradable Nanoparticles to Modulate Phenotypes of Antigen-Presenting Cells for Antigen-Specific Treatment of Autoimmune Disease. *Biomaterials* (2019) 222:119432. doi: 10.1016/j.biomaterials.2019.119432
46. Samstein RM, Perica K, Balderrama F, Look M, Fahmy TM. The Use of Deoxycholic Acid to Enhance the Oral Bioavailability of Biodegradable Nanoparticles. *Biomaterials* (2008) 29:703–8. doi: 10.1016/j.biomaterials.2007.10.026
47. Park JK, Utsumi T, Seo YE, Deng Y, Satoh A, Saltzman WM, et al. Cellular Distribution of Injected PLGA-Nanoparticles in the Liver. *Nanomedicine* (2016) 12:1365–74. doi: 10.1016/j.nano.2016.01.013
48. Liu Q, Wang X, Liu X, Kumar S, Gochman G, Ji Y, et al. Use of Polymeric Nanoparticle Platform Targeting the Liver to Induce Treg-Mediated Antigen-Specific Immune Tolerance in a Pulmonary Allergen Sensitization Model. *ACS Nano* (2019) 13:4778–94. doi: 10.1021/acsnano.9b01444
49. Carambia A, Freund B, Schwinge D, Bruns OT, Salmen SC, Ittrich H, et al. Nanoparticle-Based Autoantigen Delivery to Treg-Inducing Liver Sinusoidal Endothelial Cells Enables Control of Autoimmunity in Mice. *J Hepatol* (2015) 62:1349–56. doi: 10.1016/j.jhep.2015.01.006

50. Chen W, Jin W, Hardegen N, Lei KJ, Li L, Marinos N, et al. Conversion of Peripheral CD4+CD25- Naive T Cells to CD4+CD25+ Regulatory T Cells by TGF-Beta Induction of Transcription Factor Foxp3. *J Exp Med* (2003) 198:1875–86. doi: 10.1084/jem.20030152
51. Voll RE, Herrmann M, Roth EA, Stach C, Kalden JR, Girkontaite I. Immunosuppressive Effects of Apoptotic Cells. *Nature* (1997) 390:350–1. doi: 10.1038/37022
52. Fadok VA, Bratton DL, Konowal A, Freed PW, Westcott JY, Henson PM. Macrophages That Have Ingested Apoptotic Cells *In Vitro* Inhibit Proinflammatory Cytokine Production Through Autocrine/Paracrine Mechanisms Involving TGF-Beta, PGE2, and PAF. *J Clin Invest* (1998) 101:890–8. doi: 10.1172/JCI1112
53. Getts DR, Turley DM, Smith CE, Harp CT, McCarthy D, Feeney EM, et al. Tolerance Induced by Apoptotic Antigen-Coupled Leukocytes Is Induced by PD-L1+ and IL-10-Producing Splenic Macrophages and Maintained by T Regulatory Cells. *J Immunol* (2011) 187:2405–17. doi: 10.4049/jimmunol.1004175
54. Getts DR, Martin AJ, McCarthy DP, Terry RL, Hunter ZN, Yap WT, et al. Microparticles Bearing Encephalitogenic Peptides Induce T-Cell Tolerance and Ameliorate Experimental Autoimmune Encephalomyelitis. *Nat Biotechnol* (2012) 30:1217–24. doi: 10.1038/nbt.2434
55. Getts DR, Shea LD, Miller SD, King NJ. Harnessing Nanoparticles for Immune Modulation. *Trends Immunol* (2016) 37:715. doi: 10.1016/j.it.2015.05.007
56. Pearson RM, Casey LM, Hughes KR, Wang LZ, North MG, Getts DR, et al. Controlled Delivery of Single or Multiple Antigens in Tolerogenic Nanoparticles Using Peptide-Polymer Bioconjugates. *Mol Ther J Am Soc Gene Ther* (2017) 25:1655–64. doi: 10.1016/j.ymthe.2017.04.015
57. Rodriguez-Fernandez S, Pujol-Autonell I, Brianso F, Perna-Barrull D, Cano-Sarabia M, Garcia-Jimeno S, et al. Phosphatidylserine-Liposomes Promote Tolerogenic Features on Dendritic Cells in Human Type 1 Diabetes by Apoptotic Mimicry. *Front Immunol* (2018) 9:253. doi: 10.3389/fimmu.2018.00253
58. Casey LM, Pearson RM, Hughes KR, Liu JMH, Rose JA, North MG, et al. Conjugation of Transforming Growth Factor Beta to Antigen-Loaded Poly (Lactide- Co-Glycolide) Nanoparticles Enhances Efficiency of Antigen-Specific Tolerance. *Bioconjug Chem* (2018) 29:813–23. doi: 10.1021/acs.bioconjchem.7b00624
59. Kim SH, Moon JH, Jeong SU, Jung HH, Park CS, Hwang BY, et al. Induction of Antigen-Specific Immune Tolerance Using Biodegradable Nanoparticles Containing Antigen and Dexamethasone. *Int J Nanomed* (2019) 14:5229–42. doi: 10.2147/IJN.S210546
60. Yeste A, Takenaka MC, Mascanfroni ID, Nadeau M, Kenison JE, Patel B, et al. Tolerogenic Nanoparticles Inhibit T Cell-Mediated Autoimmunity Through SOCS2. *Sci Signal* (2016) 9:ra61. doi: 10.1126/scisignal.aad0612
61. van de Laar L, van den Bosch A, van der Kooij SW, Janssen HL, Coffer PJ, van Kooten C, et al. A Nonredundant Role for Canonical NF-kappaB in Human Myeloid Dendritic Cell Development and Function. *J Immunol* (2010) 185:7252–61. doi: 10.4049/jimmunol.1000672
62. Sun SC, Chang JH, Jin J. Regulation of Nuclear Factor-Kappab in Autoimmunity. *Trends Immunol* (2013) 34:282–9. doi: 10.1016/j.it.2013.01.004
63. Yeste A, Nadeau M, Burns EJ, Weiner HL, Quintana FJ. Nanoparticle-Mediated Codelivery of Myelin Antigen and a Tolerogenic Small Molecule Suppresses Experimental Autoimmune Encephalomyelitis. *Proc Natl Acad Sci USA* (2012) 109:11270–5. doi: 10.1073/pnas.1120611109
64. Kenison JE, Jhaveri A, Li Z, Khadse N, Tjon E, Tezza S, et al. Tolerogenic Nanoparticles Suppress Central Nervous System Inflammation. *Proc Natl Acad Sci USA* (2020) 117:32017–28. doi: 10.1073/pnas.2016451117
65. Sauer S, Bruno L, Hertweck A, Finlay D, Leleu M, Spivakov M, et al. T Cell Receptor Signaling Controls Foxp3 Expression Via PI3K, Akt, and mTOR. *Proc Natl Acad Sci USA* (2008) 105:7797–802. doi: 10.1073/pnas.0800928105
66. Long SA, Buckner JH. Combination of Rapamycin and IL-2 Increases De Novo Induction of Human CD4+CD25+FOXP3+ T Cells. *J Autoimmun* (2008) 30:293–302. doi: 10.1016/j.jaut.2007.12.012
67. Maldonado RA, LaMothe RA, Ferrari JD, Zhang AH, Rossi RJ, Kolte PN, et al. Polymeric Synthetic Nanoparticles for the Induction of Antigen-Specific Immunological Tolerance. *Proc Natl Acad Sci USA* (2015) 112:E156–65. doi: 10.1073/pnas.1408686111
68. LaMothe RA, Kolte PN, Vo T, Ferrari JD, Gelsinger TC, Wong J, et al. Tolerogenic Nanoparticles Induce Antigen-Specific Regulatory T Cells and Provide Therapeutic Efficacy and Transferrable Tolerance Against Experimental Autoimmune Encephalomyelitis. *Front Immunol* (2018) 9:281. doi: 10.3389/fimmu.2018.00281
69. Kishimoto TK. Development of ImmTOR Tolerogenic Nanoparticles for the Mitigation of Anti-Drug Antibodies. *Front Immunol* (2020) 11:969. doi: 10.3389/fimmu.2020.00969
70. Engman C, Wen Y, Meng WS, Bottino R, Trucco M, Giannoukakis N. Generation of Antigen-Specific Foxp3+ Regulatory T-Cells In Vivo Following Administration of Diabetes-Reversing Tolerogenic Microspheres Does Not Require Provision of Antigen in the Formulation. *Clin Immunol* (2015) 160:103–23. doi: 10.1016/j.clim.2015.03.004
71. Koga T, Mizui M, Yoshida N, Otomo K, Lieberman LA, Crispin JC, et al. Kn-93, an Inhibitor of Calcium/Calmodulin-Dependent Protein Kinase IV, Promotes Generation and Function of Foxp3+ Regulatory T Cells in MRL/lpr Mice. *Autoimmunity* (2014) 47:445–50. doi: 10.3109/08916934.2014.915954
72. Otomo K, Koga T, Mizui M, Yoshida N, Kriegel C, Bickerton S, et al. Cutting Edge: Nanogel-Based Delivery of an Inhibitor of CaMK4 to CD4+ T Cells Suppresses Experimental Autoimmune Encephalomyelitis and Lupus-Like Disease in Mice. *J Immunol* (2015) 195:5533–7. doi: 10.4049/jimmunol.1501603
73. Maeda K, Otomo K, Yoshida N, Abu-Asab MS, Ichinose K, Nishino T, et al. CaMK4 Compromises Podocyte Function in Autoimmune and Nonautoimmune Kidney Disease. *J Clin Invest* (2018) 128:3445–59. doi: 10.1172/JCI99507
74. Comte D, Karampetsou MP, Tsokos GC. T Cells as a Therapeutic Target in SLE. *Lupus* (2015) 24:351–63. doi: 10.1177/0961203314556139
75. Li H, Tsokos MG, Bickerton S, Sharabi A, Li Y, Moulton VR, et al. Precision DNA Demethylation Ameliorates Disease in Lupus-Prone Mice. *JCI Insight* (2018) 3:e120880. doi: 10.1172/jci.insight.120880
76. Pang L, Macauley MS, Arlian BM, Nycholat CM, Paulson JC. Encapsulating an Immunosuppressant Enhances Tolerance Induction by Siglec-Engaging Tolerogenic Liposomes. *Chembiochem* (2017) 18:1226–33. doi: 10.1002/cbic.201600702
77. Macauley MS, Pfrengle F, Rademacher C, Nycholat CM, Gale AJ, von Drygalski A, et al. Antigenic Liposomes Displaying CD22 Ligands Induce Antigen-Specific B Cell Apoptosis. *J Clin Invest* (2013) 123:3074–83. doi: 10.1172/JCI69187
78. Treiber T, Treiber N, Meister G. Regulation of microRNA Biogenesis and Its Crosstalk With Other Cellular Pathways. *Nat Rev Mol Cell Biol* (2019) 20:5–20. doi: 10.1038/s41580-018-0059-1
79. Pan W, Zhu S, Dai D, Liu Z, Li D, Li B, et al. MiR-125a Targets Effector Programs to Stabilize Treg-Mediated Immune Homeostasis. *Nat Commun* (2015) 6:7096. doi: 10.1038/ncomms8096
80. Zhang J, Chen C, Fu H, Yu J, Sun Y, Huang H, et al. Microrna-125a-Loaded Polymeric Nanoparticles Alleviate Systemic Lupus Erythematosus by Restoring Effector/Regulatory T Cells Balance. *ACS Nano* (2020) 14:4414–29. doi: 10.1021/acsnano.9b09998
81. Cheng P, Wang J. The Potential of Circulating microRNA-125a and microRNA-125b as Markers for Inflammation and Clinical Response to Infliximab in Rheumatoid Arthritis Patients. *J Clin Lab Anal* (2020) 27:e23329. doi: 10.1002/jcla.23329
82. Li S, Zhao D, Cui J, Wang L, Ma X, Li Y. Correlation of microRNA-125a/b With Acute Respiratory Distress Syndrome Risk and Prognosis in Sepsis Patients. *J Clin Lab Anal* (2020) 34:e23098. doi: 10.1002/jcla.23098
83. Steenblock ER, Fadel T, Labowsky M, Pober JS, Fahmy TM. An Artificial Antigen-Presenting Cell With Paracrine Delivery of IL-2 Impacts the Magnitude and Direction of the T Cell Response. *J Biol Chem* (2011) 286:34883–92. doi: 10.1074/jbc.M111.276329
84. Park J, Gao W, Whiston R, Strom TB, Metcalfe S, Fahmy TM. Modulation of CD4+ T Lymphocyte Lineage Outcomes with Targeted, Nanoparticle-Mediated Cytokine Delivery. *Mol Pharm* (2011) 8:143–52. doi: 10.1021/mp100203a
85. Rittchen S, Boyd A, Burns A, Park J, Fahmy TM, Metcalfe S, et al. Myelin Repair In Vivo Is Increased by Targeting Oligodendrocyte Precursor Cells With Nanoparticles Encapsulating Leukaemia Inhibitory

Factor (LIF). *Biomaterials* (2015) 56:78–85. doi: 10.1016/j.biomaterials.2015.03.044
86. De la Flor E RJ, Shevchenko RV, Alavijeh M. Multiple Sclerosis: Lifnano-CD4 for Trojan Horse Delivery of the Neuro-Protective Biologic "Lif" Into the Brain: Preclinical Proof of Concept. *Front Med Technol* (2021) 3:640569. doi: 10.3389/fmedt.2021.640569
87. Zorn E, Nelson EA, Mohseni M, Porcheray F, Kim H, Litsa D, et al. IL-2 Regulates FOXP3 Expression in Human CD4+CD25+ Regulatory T Cells Through a STAT-Dependent Mechanism and Induces the Expansion of These Cells In Vivo. *Blood* (2006) 108:1571–9. doi: 10.1182/blood-2006-02-004747
88. Zheng SG, Wang JH, Koss MN, Quismorio F Jr, Gray JD, Horwitz DA. CD4 + and CD8+ Regulatory T Cells Generated *Ex Vivo* With IL-2 and TGF-Beta Suppress a Stimulatory Graft-Versus-Host Disease With a Lupus-Like Syndrome. *J Immunol* (2004) 172:1531–9. doi: 10.4049/jimmunol.172.3.1531
89. Gray JD, Hirokawa M, Horwitz DA. The Role of Transforming Growth Factor Beta in the Generation of Suppression: An Interaction Between CD8+ T and NK Cells. *J Exp Med* (1994) 180:1937–42. doi: 10.1084/jem.180.5.1937
90. Giang S, H. D. A., Bickerton S, Fahmy TM, La Cava A. Nanoparticles Engineered as Artificial Antigen-Presenting Cells Induce Human CD4+ and CD8+ Tregs That Are Functional in Humanized Mice. *Front Immunol* (2021) 12:628059. doi: 103389/fimmu.2021.628059
91. Tsai S, Shameli A, Yamanouchi J, Clemente-Casares X, Wang J, Serra P, et al. Reversal of Autoimmunity by Boosting Memory-Like Autoregulatory T Cells. *Immunity* (2010) 32:568–80. doi: 10.1016/j.immuni.2010.03.015
92. Clemente-Casares X, Blanco J, Ambalavanan P, Yamanouchi J, Singha S, Fandos C, et al. Expanding Antigen-Specific Regulatory Networks to Treat Autoimmunity. *Nature* (2016) 530:434–40. doi: 10.1038/nature16962
93. Umeshappa CS, Singha S, Blanco J, Shao K, Nanjundappa RH, Yamanouchi J, et al. Suppression of a Broad Spectrum of Liver Autoimmune Pathologies by Single Peptide-MHC-Based Nanomedicines. *Nat Commun* (2019) 10:2150. doi: 10.1038/s41467-019-09893-5
94. Pei W, Wan X, Shahzad KA, Zhang L, Song S, Jin X, et al. Direct Modulation of Myelin-Autoreactive CD4+ and CD8+ T Cells in EAE Mice by a Tolerogenic Nanoparticle Co-Carrying Myelin Peptide-Loaded Major Histocompatibility Complexes, CD47 and Multiple Regulatory Molecules. *Int J Nanomed* (2018) 13:3731–50. doi: 10.2147/IJN.S164500
95. Shahzad KA, Wan X, Zhang L, Pei W, Zhang A, Younis M, et al. On-Target and Direct Modulation of Alloreactive T Cells by a Nanoparticle Carrying MHC Alloantigen, Regulatory Molecules and CD47 in a Murine Model of Alloskin Transplantation. *Drug Deliv* (2018) 25:703–15. doi: 10.1080/10717544.2018.1447049
96. Horwitz DA, Liu A, Bickerton S, Castaldo G, Matarese G, Fahmy TM, et al. Anti-CD2 Antibody-Coated Nanoparticles Containing Il-2 Induce Nk Cells That Protect Lupus Mice Via a TGF-Beta-Dependent Mechanism. *Front Immunol* (2020) 11:583338. doi: 10.3389/fimmu.2020.583338
97. Gray JD, Hirokawa M, Ohtsuka K, Horwitz DA. Generation of an Inhibitory Circuit Involving CD8+ T Cells, IL-2, and NK Cell-Derived TGF-Beta: Contrasting Effects of Anti-CD2 and Anti-CD3. *J Immunol* (1998) 160:2248–54.
98. Malathi S, Nandhakumar P, Pandiyan V, Webster TJ, Balasubramanian S. Novel PLGA-Based Nanoparticles for the Oral Delivery of Insulin. *Int J Nanomed* (2015) 10:2207–18. doi: 10.2147/IJN.S67947
99. Chen Y, Wu J, Wang J, Zhang W, Xu B, Xu X, et al. Targeted Delivery of Antigen to Intestinal Dendritic Cells Induces Oral Tolerance and Prevents Autoimmune Diabetes in NOD Mice. *Diabetologia* (2018) 61:1384–96. doi: 10.1007/s00125-018-4593-3
100. Koprivica I, Gajic D, Saksida T, Cavalli E, Auci D, Despotovic S, et al. Orally Delivered All-Trans-Retinoic Acid- and Transforming Growth Factor-Beta-Loaded Microparticles Ameliorate Type 1 Diabetes in Mice. *Eur J Pharmacol* (2019) 864:172721. doi: 10.1016/j.ejphar.2019.172721
101. Mitragotri S, Burke PA, Langer R. Overcoming the Challenges in Administering Biopharmaceuticals: Formulation and Delivery Strategies. *Nat Rev Drug Discov* (2014) 13:655–72. doi: 10.1038/nrd4363
102. Lee J.S HP, Chuadury R, Khan S, Bickerton S, McHugh MD, Park HB, et al. Orally Administered Bile Acid Polymer Nanoparticles for Multimodal Glucose Control and Immunotherapy of Diabetes. *Nat Biomed Eng* In press.
103. Feng J, Markwalter CE, Tian C, Armstrong M, Prud'homme RK. Translational Formulation of Nanoparticle Therapeutics From Laboratory Discovery to Clinical Scale. *J Transl Med* (2019) 17:200. doi: 10.1186/s12967-019-1945-9
104. Furst DE, Pangan AL, Harrold LR, Chang H, Reed G, Kremer JM, et al. Greater Likelihood of Remission in Rheumatoid Arthritis Patients Treated Earlier in the Disease Course: Results From the Consortium of Rheumatology Researchers of North America Registry. *Arthritis Care Res (Hoboken)* (2011) 63:856–64. doi: 10.1002/acr.20452
105. Rosenzwajg M, Lorenzon R, Cacoub P, Pham HP, Pitoiset F, El Soufi K, et al. Immunological and Clinical Effects of Low-Dose Interleukin-2 Across 11 Autoimmune Diseases in a Single, Open Clinical Trial. *Ann Rheum Dis* (2019) 78:209–17. doi: 10.1136/annrheumdis-2018-214229

Conflict of Interest: DAH is the Founder of General Nanotherapeutics, LLC and has a financial interest in the Company.

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.

frontiers in Immunology

REVIEW
published: 16 July 2021
doi: 10.3389/fimmu.2021.654701

Potential for Antigen-Specific Tolerizing Immunotherapy in Systematic Lupus Erythematosus

Sean Robinson[1] *and Ranjeny Thomas*[2]*

[1] School of Medicine, Faculty of Medicine and Biomedical Sciences, University of Queensland, St Lucia, QLD, Australia, [2] University of Queensland Diamantina Institute, The University of Queensland, Princess Alexandra Hospital, Woolloongabba, QLD, Australia

Systemic lupus erythematosus (SLE) is a chronic complex systemic autoimmune disease characterized by multiple autoantibodies and clinical manifestations, with the potential to affect nearly every organ. SLE treatments, including corticosteroids and immunosuppressive drugs, have greatly increased survival rates, but there is no curative therapy and SLE management is limited by drug complications and toxicities. There is an obvious clinical need for safe, effective SLE treatments. A promising treatment avenue is to restore immunological tolerance to reduce inflammatory clinical manifestations of SLE. Indeed, recent clinical trials of low-dose IL-2 supplementation in SLE patients showed that *in vivo* expansion of regulatory T cells (Treg cells) is associated with dramatic but transient improvement in SLE disease markers and clinical manifestations. However, the Treg cells that expanded were short-lived and unstable. Alternatively, antigen-specific tolerance (ASIT) approaches that establish long-lived immunological tolerance could be deployed in the context of SLE. In this review, we discuss the potential benefits and challenges of nanoparticle ASIT approaches to induce prolonged immunological tolerance in SLE.

Keywords: systemic lupus erythematosis, tolerance, dendritic cells, antigen (Ag), immunotherapies and vaccines

OPEN ACCESS

Edited by:
Ciriaco A Piccirillo, McGill University, Canada

Reviewed by:
Matthew Cook, Australian National University, Australia
SunAh Kang, University of North Carolina at Chapel Hill, United States

***Correspondence:**
Ranjeny Thomas
ranjeny.thomas@uq.edu.au

Specialty section:
This article was submitted to Immunological Tolerance and Regulation, a section of the journal Frontiers in Immunology

Received: *17 January 2021*
Accepted: *25 June 2021*
Published: *16 July 2021*

Citation:
Robinson S and Thomas R (2021) Potential for Antigen-Specific Tolerizing Immunotherapy in Systematic Lupus Erythematosus. Front. Immunol. 12:654701. doi: 10.3389/fimmu.2021.654701

INTRODUCTION

Systemic Lupus Erythematosus (SLE) is a chronic autoimmune inflammatory disease that affects multiple organ systems. Clinical symptoms are heterogenous and range from mild to life threatening. SLE has a significant disease burden worldwide. Mortality in SLE has decreased significantly in the past 50 years (1), attributed to the use of immunosuppressive drugs, better supportive treatments and earlier diagnosis. Acute SLE-related mortality is usually due to uncontrolled inflammation and acute renal failure, while late mortality is linked to cardiovascular complications (2). Since the 1990's late-phase clinical trials from more than 40 agents have failed in SLE. However, improvement in outcome measures, the efficacy of B cell activating factor (BAFF) and type 1 interferon (IFN) receptor 1 inhibition, and the promise of tolerance restoration, through drugs such as low-dose (LD) IL-2, underpin new optimism for future drug development (3–5). Tolerizing immunotherapies have the potential to revolutionize the treatment of autoimmune diseases by directly impacting adaptive immunity and restricting

autoinflammatory responses by inducing peripheral immunological tolerance, either by expanding pre-existing regulatory T cells (Treg) or by reprogramming autoreactive CD4+ T cells into Treg. While not extensively trialed in SLE yet, promising data in other autoimmune diseases provide learnings that may be applicable in SLE and patients at high-risk. In this review we examine the potential for antigen-specific immunotherapy to restore tolerance in lupus autoimmunity and discuss the advantages and challenges of immunotherapies and tolerizing approaches in SLE.

CLINICAL AND ETIOLOGICAL CONSIDERATIONS

SLE is 43.9% heritable, and the relative risk for siblings is 23.7. Shared environmental factors - such as infections - account for 25.8% of risk: the relative risk for spouses is 4.4 (6). Although the pathogenesis of SLE is not fully understood, the key elements are: dysregulated immune tolerance towards autologous nucleic acids with concurrent production of autoantibodies and autoreactive T-cells, disrupted clearance of apoptotic debris with increased self-antigen load and presentation to T cells, and interferon-driven inflammatory responses (7). Tissue damage – to skin, respiratory, renal, cardiovascular, central nervous and musculoskeletal systems – results from pathogenic autoantibodies, immune complex deposition and inflammation. SLE-associated environmental stressors, including UV light and infections may increase apoptotic load. With inadequate clearance, Toll Like Receptors (TLRs) recognize cellular debris (through damage associated molecular patterns, DAMPs) and initiate the inflammatory cascade, with pro inflammatory cytokine and type 1 interferon (IFN) production (8, 9). Presentation of nuclear self-antigens, such as dsDNA, chromatin, and RNA-containing antigens, to T and B cells induces the production of nuclear antigen-specific autoantibodies and autoreactive T-cells. There are multiple autoantibodies in SLE, including those directed towards nuclear antigen (ANA), double-stranded DNA (dsDNA), Smith (Sm), Ro, La, antiphospholipid (APL), and ribonucleoproteins (RNP) (10). Multiple lines of enquiry demonstrate loss of T and B cell tolerance in lupus. For example, the study of rare genetic variants associated with familial aggregation of lupus with other rheumatic autoimmune diseases identified regulation of T cell activation and T cell receptor (TCR) signaling as key underlying pathways (11). Furthermore, single cell transcriptomic analysis of peripheral blood (PB) identified antigen presenting cell, B cell and T cell dysregulation (12).

OPPORTUNITIES FOR INTERVENTION WITH TOLERIZING APPROACHES

SLE is classified (EULAR/ACR 2019 criteria) by the presence of ANA >1:80 and weighted scores for clinical and serological parameters (13). Some ANA+ individuals with very early disease or disease in evolution may fall below classification threshold. They may progress, to be re-classified as SLE, or may follow a milder and more stable clinical course. By the time of diagnosis, the majority of patients that meet SLE criteria will have some type of irreversible organ damage with clinical complications. The lupus disease course is characterized by flares and ongoing organ damage (14). Therapeutic intervention to a target of low disease activity (LLDAS) or clinical and serological remission reduces lupus-associated flares and organ damage, even when only achieved transiently (15). Typically, phase 3 trials of novel agents in SLE have struggled with small effect sizes due to disease heterogeneity, trial design issues, use of concomitant immunosuppression and endpoint validation (15). Instigation of trials in early disease and high-risk subjects not yet classified as SLE may improve the capacity to discriminate responses in patients with minimal organ damage. In a landmark phase 2 trial, a short course of T cell tolerizing immunotherapy Teplizumab, halved the progression of high-risk individuals to type 1 diabetes (16), while it had failed to meet its primary end-point in a phase 3 trial in recent-onset diabetes (17). Thus, T cell immunotherapy in people at risk (18) may be more effective before substantial organ damage.

SLE is associated with more autoantibodies than any other autoimmune disorder (19). Even before the development of disease pathology and symptoms, the pre-clinical phase is characterized by increased levels of autoantibodies, followed by a shift to multiple pathogenic autoantibodies associated with kidney, joint, heart, brain, skin and hematopoietic damage, including ANA, anti-dsDNA, anti-Sm, anti RNP, anti-APL, anti-Ro and anti-La (10). In general, anti-Ro, anti-La, and APL appear several years before the diagnosis of SLE, even in otherwise healthy individuals (20). In contrast, anti-dsDNA, anti-Sm, and anti–nuclear RNP antibodies usually appear only months before the clinical manifestations of SLE and are rarely present in healthy individuals (21, 22). In a retrospective study of 130 military personnel, use of hydroxychloroquine prior to SLE diagnosis delayed the onset of classified SLE and reduced the number of autoantibody specificities at and after diagnosis (23). At least 80% of individuals in this group met at least one SLE criterion prior to diagnosis. These results support a case for earlier therapeutic intervention with treatments of low toxicity before SLE classification and stratification of patients based on likelihood to respond. For example, current smoking was associated with elevated BAFF and reduced IL-10, particularly in ANA+ women (24). T cell expansion and type 1 IFN signatures were associated with a diagnosis of SLE in ANA+ individuals (25). Longitudinal cohort studies mapping the progression of SLE in auto-antibody positive healthy at-risk subjects will help identify early biomarkers of progression from autoantibodies to SLE, such as markers of functional loss of immune tolerance (26). Furthermore certain immune phenotypes might also be useful response biomarkers in mechanistic trials of immune tolerizing immunotherapies in individuals at high risk or with early disease.

Alternatively tolerizing approaches could be used to increase the likelihood that immunosuppressive drugs can be safely withdrawn without flare. In the BOLD clinical trial, standard immunosuppressive drugs were withdrawn and steroids substituted until flare, followed by reinstatement of standard therapy. During each phase cytokines and gene expression were analyzed to assess drug mechanism of action relative to baseline type 1 IFN transcriptomic signature. The authors identified that IL-17, IL-23 and BLyS pathways were changing with disease state and that IFN signature influenced the response of these pathways to individual drugs (27). This study provides an interesting proof-of-concept for a mechanistic trial of agents, such as tolerizing therapies, that could be introduced to reduce flare upon drug withdrawal. Although no cellular markers were included in this study, it demonstrates the utility of baseline IFN signature to stratify immune biomarker response outcomes. Future trials might also include Treg or T cell proliferation biomarkers. In this regard, a PB single cell transcriptomic resource shows co-clustering of a Treg T cell signature with dendritic cells (DC) lacking IFN-stimulated genes in lupus patients and healthy donors (12).

Mechanisms of Immune Tolerance

Immunological tolerance is a vital aspect of a healthy immune system as it allows for appropriate immune responses to infectious and tumor antigens while containing potentially damaging immune responses to self-antigen and healthy tissue. Reviews of B and T cell antigen recognition and maturation can be found here (28, 29). During development, highly self-reactive T cells in the thymus are controlled by deletion (negative selection) of T cells with the highest affinity TCR for self-peptides, and by differentiation into CD4+ CD25+ FOXP3+ Treg cells (for non-deleted autoreactive CD4+ T cells), known as central tolerance. As negative selection depends on a TCR affinity threshold, weakly autoreactive T cells circulate in the periphery (30). Peripheral T cell tolerance mechanisms control autoreactive T cells through anergy (chronic antigen exposure deactivating T cell function), deletion, and regulation by Treg (derived from thymus or generated in the periphery). Antigen-specific Treg cells can suppress activation, proliferation and cytokine production of CD4+ T cells and CD8+ T cells through interaction with APCs, including B cells and dendritic cells (DCs), presenting cognate antigen. Functional antigen-specific peripheral Treg are key to restoration of immunological tolerance with immunotherapy as they can be induced from diverse T cell precursors, and their autoantigen specificity avoids generalized immune suppression (31).

Peripherally derived Treg cells, including IL-10+ type 1 regulatory T (Tr1) cells, are promising targets for immunotherapy to counteract established autoimmune diseases. Tr1 cells are induced in the periphery, predominantly from memory CD4 T cells, and are thus an important potential target for antigen-specific tolerance approaches (32–34). They are characterized by expression of IL-10, IFN-γ and TGF-β, lack of FOXP3 expression, expression of surface markers LAG3 and CD49b, and transcription factors EOMES and Tbet (35–37). With ongoing signaling by tolerogenic APCs presenting cognate peptide. Tr1 cells are long-lived, and associated with prolonged tolerance in multiple human autoimmune conditions (38–41).

DCs comprise a heterogeneous group of phagocytic APCs that sample soluble or apoptotic antigen at skin and mucosal surfaces, and process and present antigenic peptides to T cells in draining lymph nodes in context of MHC molecules. During an inflammatory episode, e.g. driven by infection, adjuvants, or damage, pathogen- or damage-associated molecular patterns (PAMPs, DAMPs) trigger the activation of the NF-κB pathway in DCs, enhancing their capacity to stimulate naïve T cells (42). DCs presenting antigens in the presence of regulatory signals that inhibit NF-κB, such as TGF-β or immunomodulatory drugs, skew antigen-specific T-cells towards regulation (43).

DC subsets developing from hematopoietic progenitors in bone marrow include plasmacytoid DC (pDCs), myeloid/conventional DC1 (cDC1) and myeloid/conventional DC2 (cDC2), based on surface markers and immune functions (44, 45). cDC1 and moDCs can cross-present antigens derived from tissues – including viral, tumor and self-antigens – to CD8 and CD4 T cells in context of MHC I and II (46, 47). cDC2 are potent activators of naïve T cells and induce CD4+ Th1, Th2, and Th17 responses (48, 49). pDCs produce high levels of type 1 IFN in response to nucleic acids *via* TLR7 and TLR9 signaling (50, 51). In SLE, pDCs produce high levels of type 1 IFN in response to nucleic acid and nuclear antigen (52). DCs are potential targets for immunotherapies to restore the dysregulated SLE immune system. For example, crosstalk between Treg cells and DCs through cell and cytokine signaling, controls DC activation and effector T cell activation. The signaling pathways for DC development and activation are crucial when considering drug cargo in the development of novel therapies in lupus.

Pathogenesis of SLE

A basic understanding of the pathogenesis of SLE underpins a discussion on the development and effectiveness of novel immunotherapeutic agents. Here we highlight a few important factors that point to the underlying causes of SLE and that could be targeted in a therapeutic approach. For further reading please refer to the following reviews (53, 54).

SLE has been extensively studied using mouse models, which has helped illuminate pathogenesis. Some mouse models are genetically predisposed to the development of a lupus-like disease. Alternatively, a lupus-like disease may be induced in previously healthy mice. However, although spontaneous SLE models have been used to test potential therapeutics, successes in mouse models have not translated well in human trials. The NZB/NZWF1 (BW) mice and related strains develop spontaneous immune complex-mediated glomerulonephritis and mild vasculitis, with autoantibodies (anti-nuclear antibodies (ANA) and anti-dsDNA predominantly) (55). The MRL/*lpr* mouse is a unique spontaneous lupus mouse model that produces a variety of autoantibodies (ANA, anti-dsDNA, anti-Sm, anti-Ro and anti-La) and develops arthritis, cerebritis, dermatitis, vasculitis, and glomerulonephritis (56, 57). In induced mouse models of SLE, exogenous irritants or antigens are administered to replicate an environmental trigger (58, 59). Knock-out and knock-in mice backcrossed to lupus-susceptible

backgrounds has expanded understanding of signaling cascades crucial for the development of SLE (60). Few antigens have been described in lupus mouse models, hampering the testing of antigen-specific approaches for lupus in general. However, antigen non-specific tolerizing approaches have been demonstrated to improve SLE disease manifestations in mouse models (61).

Hormones, smoking, ultraviolet light, and viral/bacterial infections are classic examples of exposures triggering SLE (62, 63). Estrogen and prolactin have been shown to drive immune responses underpin in part preponderance of women with SLE (64). Viral infection such as Epstein-Barr virus and cytomegalovirus have been suspected to play a triggering role in SLE pathogenesis whereas some pathogens have been linked to a protective role in SLE (65–67). Circulating levels of lipopolysaccharides have been shown to be elevated in SLE patients and to be correlated with disease severity, presumably through cytokine production (68, 69). Recently, alterations in gut microbiome have been linked to SLE disease status (70–72). This review will not thoroughly cover these environmental factors of but we note that they are important considerations when developing therapeutic trials for potential interventions.

Apoptotic Clearance, TLRs, Nucleic Acid Sensors and Cytokines

Abnormal apoptotic clearance can trigger TLRs and nucleic acid sensors on immune and non-immune cells and produce an immune response with cytokine production (7). Rare hereditary genetic mutations e.g. in *DNASE1L3* and *PRKCD* that lead to abnormal apoptotic pathways provide crucial insight into the role of apoptotic breakdown and debris clearance in SLE (73, 74). DNase I activity degrades chromatin in the apoptotic process and mice with a mutation in this enzyme had increased levels of anti-DNA antibody production (75). Smoking induces cellular damage and promotes cytokine production, and UV light enhances apoptotic turnover, and thus may increase self-antigen burden in susceptible individuals (76, 77)

Nucleic acid sensors are important surveyors of the environment and are specifically able to recognize viral infections and induce type I IFN production. Toll-Like receptors 3, 7, 8, and 9 shape the immune response by sensing cellular debris (78). In a pristane-induced lupus mouse model, TLR7, which senses single stranded RNA, was required for RNA-reactive autoantibodies (8). TLR9 senses unmethylated CpG sequence motifs. SLE patients with active disease have higher level of TLR9+ B cells and monocytes than healthy controls, and TLR9 levels correlated with antibodies to dsDNA (79, 80).

Type I and Type II IFN contribute a large role to the pathogenesis of SLE and become elevated prior to development of autoantibodies (81). Rare single gene disorders, grouped together as Aicardi-Goutiere's syndrome, display gene defects that cause an overproduction of type I IFN (82). These patients display similar phenotypes to classic SLE, including autoantibodies.

There is a marked imbalance of T cell cytokines in SLE, with low levels of IL-2 accompanied by elevated IL-17 and IL-6 (83). IL-2 is a key cytokine in Treg development, survival and maintenance. It restricts Th17 cell development (84, 85). Elevated levels of IL-17 are thought to induce tissue inflammation and recruitment of immune cells. B cell activating factor (BAFF or BLyS), expressed by stromal and immune cells, promotes B cell activation in SLE and its levels positively correlate with antibody levels (86, 87).

Loss of Immune Tolerance

The process of autoimmune disease development can be roughly categorized into three stages: 1) a priming phase that includes an inciting event or accumulation of events in individuals at genetic and environmental risk; 2) the onset of clinical symptoms marked by organ-specific inflammation; and 3) a chronic inflammatory tissue-destructive phase (88). During the transition to clinically significant symptoms, regulatory processes, including Treg cells, fail to control pathological autoreactive B and T cells. This imbalance perpetuates the processes of bystander activation, epitope spreading and uncontrolled cytokine and antibody production. Epitope spreading involves the diversification of epitope specificity from the initial dominant epitope-specific immune response (89). The specificity of the autoimmune response spreads to include additional self-epitopes besides the initiating self-antigens. Chronic inflammation promotes tissue damage and cascading self-antigen presentation, expanding autoreactive T-cell specificities, including cryptic or sequestered epitopes (90). For example, late-stage SLE is characterized by an explosion of autoantibodies, apparently the result of chronic inflammation and epitope spreading (19). Bystander activation occurs with stress, infection or trauma-induced activation of tissue APCs, activating T cells of additional specificities, which further promote inflammation and tissue damage. Bystander T cells can provide help to B cells for autoantibody production, or to cross-presenting DCs presenting tissue-derived self-antigen (91). Treg cells may control bystander T cells and epitope spreading through interaction with cross-presenting DCs. In a rheumatoid arthritis mouse model Treg cell depletion promoted the expansion of pathogenic autoreactive T cells, an increase in inflammatory cytokines, and B-cell epitope spreading (92).

SLE is marked by abnormal B and T cell interactions and spontaneous germinal centers in secondary lymphoid organs (93–95). In SLE there is loss of functional Treg and induction of effector T cells that produce proinflammatory cytokines and BAFF, which is not normally observed in healthy people (96, 97) (98). Multiple lines of evidence demonstrate the importance of Treg in lupus pre-clinical models. In the NZB/NZWF1 spontaneous model, Treg cell adoptive transfer delayed SLE progression, reduced renal pathology, and improved survival (61), while Treg depletion accelerated disease development (99). In human SLE, most but not all studies demonstrate a reduced frequency of Treg cells (100, 101). Targeted depletion of pDCs decreased SLE-associated glomerulonephritis in mice (102, 103). In human SLE, while pDC are decreased in the blood, they are increased in lupus-affected organs, suggesting their chemo-attraction and possible expansion at these sites (104–106).

IMMUNOLOGICAL TOLERANCE THERAPEUTICS IN SLE

Current Tolerizing Strategies for SLE

There are multiple potential targeted immunotherapies undergoing research and development and early phase clinical trials for SLE (107, 108). Most techniques exploit antigen-presentation pathways of APCs or attempt to deliver antigenic cargos to locations thought to be involved in regulatory T-cell formation (109). Other strategies target antigen-specific T-cells to re-program pathogenic autoreactivity into disease-suppressing autoregulation (110, 111). **Table 1** outlines some promising therapeutic directions aiming to enhance immune tolerance by targeting DCs and Treg cells.

Expanded Treg Cell Transfer

Several groups have developed methods to expand Treg cells *ex vivo* for reintroduction as an autologous cell therapy product. Treg cells can be isolated from peripheral blood or umbilical cord blood, but must be expanded due to their low frequency. In vitro expansion strategies include anti-CD3/CD28-coated beads, with addition of IL-2 and/or TGF-β and rapamycin (121). Proof of concept experiments in lupus-prone mice showed that *ex vivo*-expanded Treg cells suppressed glomerulonephritis and prolonged survival (61, 122). *Ex vivo*-expansion of Treg cells in the presence of immunosuppressive drugs or Treg transfer into patients on immunosuppressants can be challenging, as the drugs may hinder expansion or change function (112). Furthermore, the process requires a good manufacturing practice (GMP) environment, which is challenging and expensive. A clinical trial using *ex vivo*-expanded autologous polyclonal Treg cells in patients with autoimmune disease was terminated in November 2019 due to screen failures and low enrolment. In a case report, the treatment was shown to be safe and clinical disease activity to be stable in a single SLE patient. Infused labeled Treg cells were transiently observed in PB then in diseased SLE skin, accompanied by skewing from Th1 to Th17 immunity locally (123). Treg are highly plastic and may differentiate to Th17 in inflammatory settings and where IL-2 is limiting (124). Larger studies are needed to understand the impact of Treg therapy on disease severity.

HSCT/MSCT

Hematopoietic and/or mesenchymal stem cell transfer (HSCT and MSCT, respectively) have been trialed in patients with severe autoimmune diseases, including SLE, who have failed standard therapy. In SLE patients, HSCT has successfully induced long-term remission (125). In 15 patients with severe SLE evaluated up to 8 years after HSCT, $CD4^+CD25^{high}Foxp3^+$ Treg and $LAP^{high}TGF\text{-}\beta^+CD8^+Foxp3^+$ cells were restored to levels and function similar to healthy subjects (117). These promising results suggest that HSCT may reestablish immune tolerance by replenishing multiple types of Treg cells. However, as HSCT is associated with significant risks, treatment complications and cost, it is currently reserved for treatment-refractory patients. A 4-year follow-up of an open-label trial of MSCT in 87 treatment-refractory SLE patients found a 28% remission rate post-infusion (118). While double-blind placebo-controlled trials are needed to understand the true benefits of MSCT, these trials provide evidence that tolerance may be successfully re-established in SLE.

Low-Dose IL-2

IL-2 levels and CD25 expression by Treg are reduced in SLE patients and murine lupus models (126–128). IL-2 plays a pleiomorphic role in the immune system. One of its functions is to expand and promote survival of Treg cells (129, 130). Reduced IL-2 favors the differentiation of IFN-γ-producing Th1 and IL-17 producing Th17 cells and their accumulation in skin and kidneys (131, 132), and is associated with inflammation. In lupus-prone mice, IL-2 treatment increased levels of Treg cells in lymphoid and peripheral organs and protected them from SLE-related organ damage (99, 133). There have been several trials in lupus showing safety and Treg expansion (128, 134). In a recent double-blind placebo-controlled clinical trial in patients with suboptimally controlled SLE, LD IL-2 for 12 weeks (s.c. alternate days for three 2-week cycles), the SLE Responder Index (SRI)-4 response rates at week 12 were 55.17% and 30.00% in LD IL-2 and placebo groups respectively ($p=0.052$). Although the primary end point was not met, the significantly greater lupus nephritis complete remission rate in the LD IL-2 arm was notable. Immunologically, IL-2 supplementation significantly increased Tregs and NK cells but did not change total CD4+ or CD8+ T cells and there was no increase in viral load of pre-existing viruses (3). While promising, LD IL-2 dosing may be complicated by concomitant expansion of regulatory and cytotoxic cells. Furthermore, development of neutralizing autoantibodies with continued treatment is a potential risk (135). Targeted IL-2 therapies may allow more precise manipulation of the immune response and longer duration of action. For example, anti-CD4 and anti-CD2-coated poly(lactic-co-glycolic) acid (PLGA) nanoparticles loaded with IL-2 and TGFβ expanded Treg cells *in vitro* and *in vivo* in the BDF1 lupus pre-clinical model (136). In a recent phase 1b clinical trial of a polyethylene glycol (PEG) conjugate of IL-2 (NKTR-358) in

TABLE 1 | Treg and DC based Therapies without Autoantigen.

Therapy	Mechanism	Clinical trial for SLE	References
Adoptive Treg cell or DC transfer	Non antigen-specific increase Treg cells, Antigen-specific tolerogenic DC immunotherapy to induce Treg cells	Yes for Tregs, No for DCs	(112–116)
HSCT/MSCT	Non antigen-specific immune tolerance	Yes	(117, 118)
Low-dose IL-2	Non antigen-specific increased survival, proliferation and/or function of Treg cells	Yes	(3)
Targeted DC immunotherapy	Induce tolerance through tolerogenic antigen delivery to DCs	No	(119, 120)

patients with mild to moderate SLE, dose-dependent increases in Tregs (up to 11 fold) were observed, which returned to baseline 20-30 days post-dose (137). Anti-IL-2 antibodies were not reported.

Tolerogenic DCs

DCs play a critical role in maintaining self-tolerance. Indeed, targeting steady-state skin migratory DC with antigen coupled to DC-selective antibodies induced antigen-specific tolerance (138). Tolerogenic DCs can also be generated *in vitro* from monocytes or murine bone marrow precursors in the presence of NF-κB inhibitors 1,25 $(OH)_2$ vitamin D3 (calcitriol), rapamycin or glucocorticoids. After proof-of-concept studies in experimental animal models (139, 140), several groups translated antigen-specific immunotherapy using modified or tolerogenic autologous DCs and autoantigenic peptides to clinical trials for MS (113) and RA (114, 115). These trials demonstrate the feasibility and safety of this approach, with preliminary evidence of an immunomodulatory effect in RA. In two pre-clinical lupus models, histone antigen-loaded tolerogenic DCs improved clinical scores, increased Treg in affected skin and reduced anti-histone autoantibodies (141). Tolerogenic DCs exposed to apoptotic cells were generated from PB monocytes derived from lupus patients (142). Other approaches have been developed to target DCs directly *in situ*, including a PLGA nanogel to deliver the immunomodulator mycophenolic acid (MPA) to DCs (119, 120). DCs took up the PLGA-lipid-MPA nanogel more efficiently and with better DC suppression than a PLGA nanogel. In a murine lupus model, PLGA-MPA nanogel increased median survival by 3 months when given prophylactically and by 2 months when given to mice with advanced renal damage. Consistent with the local effects of MPA on DCs, treated mice had a substantial reduction in DC-derived inflammatory cytokines such as IFN-γ and IL-12. Although not strictly immune tolerance, this approach achieves sustained delivery of MPA to induce a prolonged anti-inflammatory effect.

Lupuzor

Lupuzor (rigerimod or IPP-201101) is a 21aa peptide representing residues 131–151 of the 70K spliceosomal protein within the U1 small nuclear RNP, phosphorylated at Ser140. This promiscuous peptide sequence was identified using *ex vivo* peptide screening techniques (143). This epitope is recognized by IgG antibodies and CD4+ T cells from H-2^k MRL/*lpr* and H-$2^{d/z}$ (NZB × NZW)F1 lupus-prone mice (143, 144). With i.v. delivery, the peptide inhibits chaperone-mediated autophagy and reduces B cell MHC class II expression (145). Two trials of IPP-201101 immunotherapy in SLE demonstrated safety and potential efficacy (146, 147). However, IPP-201101 failed to meet its primary end point of superiority over standard care in phase III clinical trials (148). The peptide seemed to have non antigen-specific immunomodulatory properties, rather than inducing antigen-specific regulation, and this may be why it was not superior to standard care. Standard of care high dose glucocorticoids and immunosuppressive drugs are likely more bioavailable than an immunosuppressive peptide.

These treatment strategies are antigen non-specific and use nanoparticles (NP) to deliver biologics or immunosuppressive drugs. In the following sections we consider antigen-specific tolerizing approaches using NP in SLE.

Potential Antigen-Specific Tolerizing Platforms for SLE

Antigen-specific therapies for autoimmune diseases involve the delivery of autoantigen in a regulatory context, with or without a delivery vehicle that reprograms APCs by modulating NF-κB, or by antigen delivery to a naturally tolerogenic site e.g. by targeting steady-state skin-draining APCs or the liver tolerogenic environment. Some approaches may directly differentiate Tr1 cells from memory T cells.

Peptide alone, delivered s.c., can be tolerogenic. For example, an islet proinsulin epitope returned promising results in phase 1 trials in T1D (149). Peptides that associate with MHC class II molecules expressed by APCs, without the need for antigen processing, can directly target steady-state DC *in vivo*. Such antigen processing independent epitopes ("apitopes") selectively bind steady-state DCs *in vivo* because steady-state DCs bear peptide receptive/empty MHC II at the cell surface, which is lost upon DC activation (150, 151). Apitopes induce tolerance through induction of anergy and generation of Tr1 cells (152). Tr1 cells selectively express a tolerance-associated set of genes (153, 154). Phase 1 and 2 clinical trials of multiple low dose apitope delivery have been undertaken in Graves' disease and MS respectively. While low-dose soluble antigen administered s.c. is non-immunogenic, high dose peptide, aggregates or protein complexes can induce an immune response through immune complex formation, macrophage or DC activation and development of autoantibodies.

NPs Delivering Antigens and Immunomodulators

Liposome formulations loaded with peptide or protein antigens and various NF-κB inhibitors, including curcumin, quecertin and BAY11-7082 induced antigen-specific tolerance in mice with antigen-induced arthritis (155). We also developed and undertook pre-clinical studies of liposomes co-encapsulating calcitriol and peptide. Calcitriol/peptide liposomes promoted the differentiation of antigen-specific $Foxp3^+$ Treg, anergy of Tmem, and IL-10 production upon restimulation with antigen *ex vivo* (156). Notably, liposomes were preferentially taken up by activated $PD\text{-}L1^+$ migratory DCs, and regulation was PD-L1-dependent. We translated this to a phase 1b clinical trial in RA. Other groups have co-encapsulated antigens in NPs with either rapamycin (157) or aryl hydrocarbon receptor (AhR) ligands (158) for *in vivo* uptake by DC. With substitution of suitable lupus antigenic peptides, these liposome or NP approaches could be adapted to lupus patients.

Nanoparticles Leveraging Natural Tolerogenic Processes

Other research groups have developed NPs that resemble apoptotic bodies, to promote a tolerogenic response to encapsulated antigen. Specifically, i.v. administration of 500nm PLGA particles encapsulating antigen induced antigen specific tolerance (159, 160). These relatively large, negatively-charged

particles are preferentially taken up by DCs and macrophages expressing MARCO, and induce antigen-specific suppression in the absence of an immunomodulatory drug (161). Another strategy to mimic signals from apoptotic bodies uses phosphatidylserine (PS) liposomes. During apoptosis, the PS phospholipid translocates from the inner leaflet to the outer leaflet of the lipid bilayer of the dying cell. PS liposomes suppressed pre-clinical models of T1D and acute EAE in a non-antigen-specific manner (162, 163). It is unclear whether this technique would succeed in SLE, which is characterized by impaired clearance of apoptotic cells.

Peptide-MHC NPs

The TCR may also be directly targeted with NPs coated with peptide loaded onto MHC class I or II, without co-stimulation. After i.v. delivery of iron oxide nanoparticles coated with peptide-MHC class I complexes (pMHC-I) they suppressed autoreactive CD8+ memory T cells and converted them to a regulatory, anergic phenotype (110). Nanoparticles coated with pMHC-II differentiated cognate autoreactive CD4 memory T cells into Tr1 cells producing IL-10 (111, 164). Nanoparticles coated with pMHC-II suppressed autoimmune symptoms in several pre-clinical models in an antigen-specific manner, without compromising systemic immunity (111). To date, this approach has not been translated to clinical trials.

Thus, a wide array of nanoparticle technologies has been developed. **Figure 1** describes some of the technologies incorporating autoantigens, immunomodulatory drugs, or targeting strategies, or a combination of strategies. In summary, approaches that promote the expansion of antigen-specific Treg cells, particularly Tr1 cells derived from autoreactive memory T cells, will be required to control bystander cytokine production and epitope spreading in multi-system autoimmune diseases, such as SLE.

AUTOANTIGENS IN SLE

Many autoantigens potentially contribute to the development of SLE and it is unclear which antigen(s) should be targeted in antigen-specific immunotherapy. Several promiscuous epitopes have been described across mice and humans. Choosing an antigen is challenging because there are many different pre-clinical lupus models, the disease is highly heterogeneous in humans, and translation of antigen discovery from mouse to human is difficult. However, assays of T cell responses in organ-dominant lupus "endotypes" may offer opportunities to identify relevant skin, joint, renal, neurological and hematologic antigenic epitopes that are suitable for clinical trials with focused outcomes.

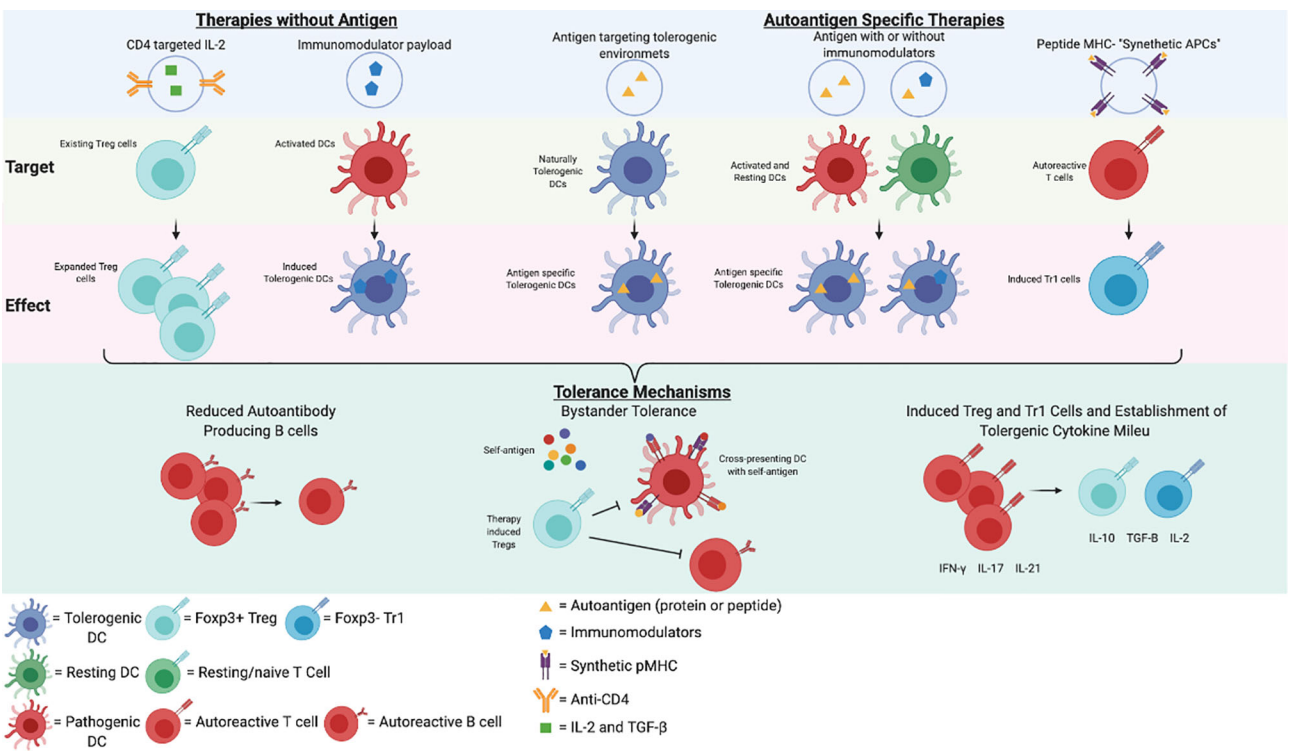


FIGURE 1 | Potential Nanoparticle Therapies in SLE. A representation of potential immunotherapies for SLE and a simplified schematic of their mechanisms of action. This figure was created in BioRender.com.

Despite these hurdles, antigen-specific immunotherapies with a single strong autoantigen that also promote bystander tolerance could leverage the expansion of antigen-specific Treg cells and the suppression of cross-presenting DCs carrying relevant epitopes from diseased tissue to draining lymph nodes. Bystander tolerance has been demonstrated for several immunotherapies in pre-clinical models, including apitopes, peptide/calcitriol liposomes, and pMHC-NP, associated with the modulation of immune responses other than the epitope included in the immunotherapy (111, 165). For example, in a type 1 diabetes mouse model, calcitriol liposomes encapsulating a single islet CD4 epitope suppressed the disease and bystander islet-reactive CD8+ cytotoxic T cells (166). The advantages of harnessing bystander tolerance mediated by Treg cells, compared to generalized immunosuppression, is that bystander suppression is tissue-restricted, and Treg develop from autoreactive memory T cells. However, suitable antigenic epitopes must be identified.

Haplotypes containing DR2/DQ6, and DR3/DQ2 alleles are associated with SLE (167). DR2/DR3 heterozygosity is associated with anti-Ro, anti-La, anti-Sm, anti-ribosomal-P or anti-ribonuclear protein antibodies, while HLA-DR homozyosity is associated with anti-Sm and anti-dsDNA (167). HLA-restriction poses a potential hurdle for the applicability of peptide-specific immunotherapies, as peptides need to be identified and matched to patient MHC class II. Long antigenic sequences or mixtures of epitopes that cover a large percentage of the diseased population provide potential solutions. HLA-restricted soluble or NP-associated peptide immunotherapy may be a good way to achieve some early positive immune outcomes of antigen-specific immunotherapies, including bystander tolerance in proof-of-concept clinical trials. Subsequently, tolerizing immunotherapies with multiple autoantigens or proteins could be further tested.

Strategies to identify potential self-peptides include: screening autoreactive T cell proliferation or cytokine production *ex vivo*, peptide elution from MHC II molecules, and autoantibody binding epitopes. Immunization studies in DR3 transgenic mice have been used to map DR3-restricted SmD T cell epitopes (168). Studies investigating apoptotic cell-derived self-epitopes recognized by pathogenic T cells in human and lupus-prone mouse models identified potential histone epitopes, including histone $H1'_{22-42}$, $H4_{16-39}$, $H4_{71-94}$ and $H3_{82-105}$ (169, 170). These extended epitopes bind multiple HLA-DR allomorphs. Most also bound anti-histone autoantibodies (171, 172). In human PB cultures, these peptides promoted TGF-β secretion and expanded Foxp3+Treg cells in the presence of IL-2 *in vitro* (170). In SVF1 lupus-prone mice, s.c. administration of $H4_{71-94}$ every 2 weeks induced TGF-β-producing pDCs and Treg cells and protected mice from renal disease (173, 174). A $70K\text{-}U1RNP_{131-151}$ T helper epitope was identified in NZBxNZW F1 and MRL/Fas(lpr) mice, which led to further identification of SmD1 and hnRNP A2/B1 epitopes in each strain. Of interest the SmD_{95-119} epitope recognized by anti-Sm antibodies is homologous to an Epstein-Barr virus EBNA I peptide, suggesting a mechanism for epitope spreading through bystander T helper cells (144, 175). Certain nuclear antigens tend to induce epitope spreading to related other nuclear antigens in mouse models (**Table 2**). If administered as antigen-specific tolerizing immunotherapy, one would therefore predict induction of bystander tolerance (183).

TABLE 2 | Epitope spreading in mouse models after autoantigen immunization.

Antigen	Autoimmune Epitope Spread	Reference
Ro 60 (aa 316–335)	Ro60, La, Sm, U1RNP	(176)
SmD1 protein	A-RNP, SmD	(176)
SmB protein	A-RNP, SmD	(176)
SmD183–119	SmD, dsDNA	(177)
SmB′/B aa PPPGMRPP	SmD, 70k-/A-U1RNP	(178)
Murine La (aa 13–30)	Ro52	(179)
A2/B1 hnRNP (aa 50–70)	hnRNP	(180)
Nucleosome (lupus-prone mice)	dsDNA, nucleosome, histone	(181)
La (aa 13–30)	La, Ro	(179)
Histone H1	H2, ssDNA	(182)

Further research into SLE immunotherapy would benefit greatly from a humanized model that could better represent the human immune system (184, 185).

CONCLUSION

SLE is a devastating autoimmune disease with a large unmet need for better therapies. Promising work has identified some immunological markers of immune tolerance in individuals at risk who have not progressed to a diagnosis of SLE, and some nuclear-derived antigenic epitopes that may be presented by multiple MHC II molecules. More work is needed to carefully map the autoantigen specificity and HLA restriction of expanded T cells in patients with recent-onset SLE. The pre-clinical phase and milder organ-specific endotypes of SLE provide potential opportunities to intervene in individuals with a less aggressive or more focused disease processes, associated with lower levels of organ damage. Technological platforms showing promise in early-phase clinical trials or preclinical models in other autoimmune diseases could be adapted for trials in SLE. Given the clinical complexity, sensible beginning strategies would comprise small mechanistic studies with immune biomarker and safety outcomes in well-defined limited disease settings.

AUTHOR CONTRIBUTIONS

SR drafted and edited manuscript, and compiled figure. RT edited manuscript, figure and tables. All authors contributed to the article and approved the submitted version.

FUNDING

This manuscript is part of a project that has received funding from the Innovative Medicines Initiative 2 Joint Undertaking under grant agreement No 777357. This Joint Undertaking receives support from the European Union's Horizon 2020 research and innovation programme and EFPIA. RT was supported by NHMRC grant 1079238 and by Arthritis Queensland.

REFERENCES

1. Bernatsky S, Boivin JF, Joseph L, Manzi S, Ginzler E, Gladman DD, et al. Mortality in Systemic Lupus Erythematosus. *Arthritis Rheumatism* (2006) 54:2550–7. doi: 10.1002/art.21955
2. Borchers AT, Keen CL, Shoenfeld Y, Gershwin ME. Surviving the Butterfly and the Wolf: Mortality Trends in Systemic Lupus Erythematosus. *Autoimmun Rev* (2004) 3:423–53. doi: 10.1016/j.autrev.2004.04.002
3. He J, Zhang R, Shao M, Zhao X, Miao M, Chen J, et al. Efficacy and Safety of Low-Dose IL-2 in the Treatment of Systemic Lupus Erythematosus: A Randomised, Double-Blind, Placebo-Controlled Trial. *Ann Rheumatic Dis* (2020) 79:141–9. doi: 10.1136/annrheumdis-2019-215396
4. Urowitz MB, Ohsfeldt RL, Wielage RC, Dever JJ, Zakerifar M, Asukai Y, et al. Comparative Analysis of Long-Term Organ Damage in Patients With Systemic Lupus Erythematosus Using Belimumab Versus Standard Therapy: A Post Hoc Longitudinal Study. *Lupus Sci Med* (2020) 7. doi: 10.1136/lupus-2020-000412
5. Morand EF, Furie R, Tanaka Y, Bruce IN, Askanase AD, Richez C, et al. Trial of Anifrolumab in Active Systemic Lupus Erythematosus. *N Engl J Med* (2020) 382:211–21. doi: 10.1056/NEJMoa1912196
6. Kuo CF, Grainge MJ, Valdes AM, See LC, Luo SF, Yu KH, et al. Familial Aggregation of Systemic Lupus Erythematosus and Coaggregation of Autoimmune Diseases in Affected Families. *JAMA Intern Med* (2015) 175:1518–26. doi: 10.1001/jamainternmed.2015.3528
7. Casciola-Rosen LA, Anhalt G, Rosen A. Autoantigens Targeted in Systemic Lupus Erythematosus Are Clustered in Two Populations of Surface Structures on Apoptotic Keratinocytes. *J Exp Med* (1994) 179:1317–30. doi: 10.1084/jem.179.4.1317
8. Savarese E, Steinberg C, Pawar RD, Reindl W, Akira S, Anders HJ, et al. Requirement of Toll-Like Receptor 7 for Pristane-Induced Production of Autoantibodies and Development of Murine Lupus Nephritis. *Arthritis Rheum* (2008) 58:1107–15. doi: 10.1002/art.23407
9. Honda K, Ohba Y, Yanai H, Negishi H, Mizutani T, Takaoka A, et al. Spatiotemporal Regulation of MyD88–IRF-7 Signalling for Robust Type-I Interferon Induction. *Nature* (2005) 434:1035–40. doi: 10.1038/nature03547
10. Arbuckle MR, McClain MT, Rubertone MV, Scofield RH, Dennis GJ, James JA, et al. Development of Autoantibodies Before the Clinical Onset of Systemic Lupus Erythematosus. *New Engl J Med* (2003) 349:1526–33. doi: 10.1056/NEJMoa021933
11. Wang CL, Kuo CF, Yeh YH, Hsieh MY, Kuo CT, Chang SH. Familial Aggregation of Myocardial Infarction and Coaggregation of Myocardial Infarction and Autoimmune Disease: A Nationwide Population-Based Cross-Sectional Study in Taiwan. *BMJ Open* (2019) 9:e023614. doi: 10.1136/bmjopen-2018-023614
12. Nehar-Belaid D, Hong S, Marches R, Chen G, Bolisetty M, Baisch J, et al. Mapping Systemic Lupus Erythematosus Heterogeneity at the Single-Cell Level. *Nat Immunol* (2020) 21:1094–106. doi: 10.1038/s41590-020-0743-0
13. Aringer M, Costenbader K, Daikh D, Brinks R, Mosca M, Ramsey-Goldman R, et al. 2019 European League Against Rheumatism/American College of Rheumatology Classification Criteria for Systemic Lupus Erythematosus. *Ann Rheum Dis* (2019) 78:1151–9. doi: 10.1136/annrheumdis-2019-216700
14. Tselios K, Gladman DD, Touma Z, Su J, Anderson N, Urowitz MB. Disease Course Patterns in Systemic Lupus Erythematosus. *Lupus* (2019) 28:114–22. doi: 10.1177/0961203318817132
15. Golder V, Tsang A.S.M.W.P.. Treatment Targets in SLE: Remission and Low Disease Activity State. *Rheumatol (Oxf)* (2020) 59:v19–28. doi: 10.1093/rheumatology/keaa420
16. Herold KC, Bundy BN, Long SA, Bluestone JA, DiMeglio LA, Dufort MJ, et al. An Anti-CD3 Antibody, Teplizumab, in Relatives at Risk for Type 1 Diabetes. *N Engl J Med* (2019) 381:603–13. doi: 10.1056/NEJMoa1902226
17. Vudattu NK, Herold KC. Treatment of New Onset Type 1 Diabetes With Teplizumab: Successes and Pitfalls in Development. *Expert Opin Biol Ther* (2014) 14:377–85. doi: 10.1517/14712598.2014.881797
18. Insel RA, Dunne JL, Atkinson MA, Chiang JL, Dabelea D, Gottlieb PA, et al. Staging Presymptomatic Type 1 Diabetes: A Scientific Statement of JDRF, the Endocrine Society, and the American Diabetes Association. *Diabetes Care* (2015) 38:1964–74. doi: 10.2337/dc15-1419
19. Sherer Y, Gorstein A, Fritzler MJ, Shoenfeld Y. Autoantibody Explosion in Systemic Lupus Erythematosus: More Than 100 Different Antibodies Found in SLE Patients. *Semin Arthritis Rheum* (2004) 34:501–37. doi: 10.1016/j.semarthrit.2004.07.002
20. Spronk PE, Limburg PC, Kallenberg CGM. Review : Serological Markers of Disease Activity in Systemic Lupus Erythematosus. *Lupus* (1995) 4:86–94. doi: 10.1177/096120339500400202
21. Gaither KK, Fox OF, Yamagata H, Mamula MJ, Reichlin M, Harley JB. Implications of Anti-Ro/Sjögren's Syndrome A Antigen Autoantibody in Normal Sera for Autoimmunity. *J Clin Invest* (1987) 79:841–6. doi: 10.1172/JCI112892
22. Ma Z, Rp M, Js M. Anti-dsDNA Antibodies in Laboratory Workers Handling Blood From Patients With Systemic Lupus Erythematosus. *J Rheumatol* (1992) 19:1380–4.
23. Dema B, Charles N. Autoantibodies in SLE: Specificities, Isotypes and Receptors. *Antibodies (Basel)* (2016) 5:2. doi: 10.3390/antib5010002
24. Hahn J, Leatherwood C, Malspeis S, Liu X, Lu B, Roberts AL, et al. Associations Between Smoking and Systemic Lupus Erythematosus (SLE)-Related Cytokines and Chemokines Among US Female Nurses. *Arthritis Care Res (Hoboken)* (2020). doi: 10.1002/acr.24370
25. Slight-Webb S, Smith M, Bylinska A, Macwana S, Guthridge C, Lu R, et al. Autoantibody-Positive Healthy Individuals With Lower Lupus Risk Display a Unique Immune Endotype. *J Allergy Clin Immunol* (2020) 146:1419–33. doi: 10.1016/j.jaci.2020.04.047
26. Li Q-Z, Karp DR, Quan J, Branch VK, Zhou J, Lian Y, et al. Risk Factors for ANA Positivity in Healthy Persons. *Arthritis Res Ther* (2011) 13:R38–8. doi: 10.1186/ar3271
27. Merrill JT, Immermann F, Whitley M, Zhou T, Hill A, O'Toole M, et al. The Biomarkers of Lupus Disease Study: A Bold Approach May Mitigate Interference of Background Immunosuppressants in Clinical Trials. *Arthritis Rheumatol* (2017) 69:1257–66. doi: 10.1002/art.40086
28. Aster JC. *Normal B and T Lymphocyte Development*. In: Rosmarin AG, editor. (2021). UpToDate, UpToDate, UpToDate.
29. Monroe JG, Rothenberg E, eds. *Molecular Biology of B-Cell and T-Cell Development*. Springer (1998).
30. Anderton SM, Wraith DC. Selection and Fine-Tuning of the Autoimmune T-Cell Repertoire. *Nat Rev Immunol* (2002) 2:487–98. doi: 10.1038/nri842
31. Haribhai D, Williams JB, Jia S, Nickerson D, Schmitt EG, Edwards B, et al. A Requisite Role for Induced Regulatory T Cells in Tolerance Based on Expanding Antigen Receptor Diversity. *Immunity* (2011) 35:109–22. doi: 10.1016/j.immuni.2011.03.029
32. Meiron M, Zohar Y, Anunu R, Wildbaum G, Karin N. CXCL12 (SDF-1α) Suppresses Ongoing Experimental Autoimmune Encephalomyelitis by Selecting Antigen-Specific Regulatory T Cells. *J Exp Med* (2008) 205:2643–55. doi: 10.1084/jem.20080730
33. Bollyky PL, Wu RP, Falk BA, Lord JD, Long SA, Preisinger A, et al. ECM Components Guide IL-10 Producing Regulatory T-Cell (TR1) Induction From Effector Memory T-Cell Precursors. *PNAS* (2011) 108:7938–43. doi: 10.1073/pnas.1017360108
34. Yao Y, Vent-Schmidt J, McGeough MD, Wong M, Hoffman HM, Steiner TS, et al. Tr1 Cells, But Not Foxp3+ Regulatory T Cells, Suppress NLRP3 Inflammasome Activation via an IL-10–Dependent Mechanism. *J Immunol* (2015) 195:488–97. doi: 10.4049/jimmunol.1403225
35. Bluestone JA, Abbas AK. Natural Versus Adaptive Regulatory T Cells. *Nat Rev Immunol* (2003) 3:253–7. doi: 10.1038/nri1032
36. Roncarolo MG, Gregori S, Bacchetta R, Battaglia M, Gagliani N. The Biology of T Regulatory Type 1 Cells and Their Therapeutic Application in Immune-Mediated Diseases. *Immunity* (2018) 49:1004–19. doi: 10.1016/j.immuni.2018.12.001
37. Zhang P, Lee JS, Gartlan KH, Schuster IS, Comerford I, Varelias A, et al. Eomesodermin Promotes the Development of Type 1 Regulatory T (TR1) Cells. *Sci Immunol* (2017) 2. doi: 10.1126/sciimmunol.aah7152
38. Bacchetta R, Bigler M, Touraine JL, Parkman R, Tovo PA, Abrams J, et al. High Levels of Interleukin 10 Production In Vivo Are Associated With Tolerance in SCID Patients Transplanted With HLA Mismatched Hematopoietic Stem Cells. *J Exp Med* (1994) 179:493–502. doi: 10.1084/jem.179.2.493
39. Gianfrani C, Levings MK, Sartirana C, Mazzarella G, Barba G, Zanzi D, et al. Gliadin-Specific Type 1 Regulatory T Cells From the Intestinal Mucosa of

Treated Celiac Patients Inhibit Pathogenic T Cells. *J Immunol* (2006) 177:4178–86. doi: 10.4049/jimmunol.177.6.4178
40. Roncarolo MG, Gregori S, Bacchetta R, Battaglia M. Tr1 Cells and the Counter-Regulation of Immunity: Natural Mechanisms and Therapeutic Applications. In: Fillatreau S, O'Garra A, editors. *Interleukin-10 in Health and Disease*. Berlin, Heidelberg: Springer (2014). p. 39–68.
41. Głobińska A, Boonpiyathad T, Satitsuksanoa P, Kleuskens M, van de Veen W, Sokolowska M, et al. Mechanisms of Allergen-Specific Immunotherapy: Diverse Mechanisms of Immune Tolerance to Allergens. *Ann Allergy Asthma Immunol* (2018) 121:306–12. doi: 10.1016/j.anai.2018.06.026
42. Mellman I. Dendritic Cells: Master Regulators of the Immune Response. *Cancer Immunol Res* (2013) 1:145–9. doi: 10.1158/2326-6066.CIR-13-0102
43. Martin E, O'Sullivan B, Low P, Thomas R. Antigen-Specific Suppression of a Primed Immune Response by Dendritic Cells Mediated by Regulatory T Cells Secreting Interleukin-10. *Immunity* (2003) 18:155–67. doi: 10.1016/S1074-7613(02)00503-4
44. Granot T, Senda T, Carpenter DJ, Matsuoka N, Weiner J, Gordon CL, et al. Dendritic Cells Display Subset and Tissue-Specific Maturation Dynamics Over Human Life. *Immunity* (2017) 46:504–15. doi: 10.1016/j.immuni.2017.02.019
45. Guilliams M, Dutertre C-A, Scott CL, McGovern N, Sichien D, Chakarov S, et al. Unsupervised High-Dimensional Analysis Aligns Dendritic Cells Across Tissues and Species. *Immunity* (2016) 45:669–84. doi: 10.1016/j.immuni.2016.08.015
46. Australian Government Department of Health. *The Pharmaceutical Benefits Scheme: Expenditure and Prescriptions Twelve Months to 30 June 2018* (2018). Available at: https://www.pbs.gov.au/info/statistics/expenditure-prescriptions/expenditure-prescriptions-twelve-months-to-30-june-2018.
47. Haniffa M, Shin A, Bigley V, McGovern N, Teo P, See P, et al. Human Tissues Contain CD141hi Cross-Presenting Dendritic Cells With Functional Homology to Mouse CD103+ Nonlymphoid Dendritic Cells. *Immunity* (2012) 37:60–73. doi: 10.1016/j.immuni.2012.04.012
48. Villani A-C, Satija R, Reynolds G, Sarkizova S, Shekhar K, Fletcher J, et al. Single-Cell RNA-Seq Reveals New Types of Human Blood Dendritic Cells, Monocytes, and Progenitors. *Science* (2017) 356:eaah4573. doi: 10.1126/science.aah4573
49. Di Blasio S, Wortel IMN, van Bladel DAG, de Vries LE, Duiveman-de Boer T, Worah K, et al. Human CD1c+ DCs Are Critical Cellular Mediators of Immune Responses Induced by Immunogenic Cell Death. *OncoImmunology* (2016) 5:e1192739. doi: 10.1080/2162402X.2016.1192739
50. Siegal FP, Kadowaki N, Shodell M, Fitzgerald-Bocarsly PA, Shah K, Ho S, et al. The Nature of the Principal Type 1 Interferon-Producing Cells in Human Blood. *Science* (1999) 284:1835. doi: 10.1126/science.284.5421.1835
51. Lee HK, Lund JM, Ramanathan B, Mizushima N, Iwasaki A. Autophagy-Dependent Viral Recognition by Plasmacytoid Dendritic Cells. *Science* (2007) 315:1398. doi: 10.1126/science.1136880
52. Liu Y-J. IPC: Professional Type 1 Interferon-Producing Cells and Plasmacytoid Dendritic Cell Precursors. *Annu Rev Immunol* (2004) 23:275–306. doi: 10.1146/annurev.immunol.23.021704.115633
53. Tsokos GC, Lo MS, Reis PC, Sullivan KE. New Insights Into the Immunopathogenesis of Systemic Lupus Erythematosus. *Nat Rev Rheumatol* (2016) 12:716–30. doi: 10.1038/nrrheum.2016.186
54. Mok CC, Lau CS. Pathogenesis of Systemic Lupus Erythematosus. *J Clin Pathol* (2003) 56:481–90. doi: 10.1136/jcp.56.7.481
55. Dixon FJ, Andrews BS, Eisenberg RA, McConahey PJ, Theofilopoulos AN, Wilson CB. Etiology and Pathogenesis of a Spontaneous Lupus-Like Syndrome in Mice. *Arthritis Rheum* (1978) 21:S64–7. doi: 10.1002/art.1780210909
56. Rauch J, Murphy E, Roths JB, Stollar BD, Schwartz RS. A High Frequency Idiotypic Marker of Anti-DNA Autoantibodies in MRL-Ipr/Ipr Mice. *J Immunol* (1982) 129:236–41.
57. Eisenberg RA, Tan EM, Dixon FJ. Presence of Anti-Sm Reactivity in Autoimmune Mouse Strains. *J Exp Med* (1978) 147:582–7. doi: 10.1084/jem.147.2.582
58. Satoh M, Reeves WH. Induction of Lupus-Associated Autoantibodies in BALB/c Mice by Intraperitoneal Injection of Pristane. *J Exp Med* (1994) 180:2341–6. doi: 10.1084/jem.180.6.2341
59. Yokogawa M, Takaishi M, Nakajima K, Kamijima R, Fujimoto C, Kataoka S, et al. Epicutaneous Application of Toll-Like Receptor 7 Agonists Leads to Systemic Autoimmunity in Wild-Type Mice: A New Model of Systemic Lupus Erythematosus. *Arthritis Rheumatol* (2014) 66:694–706. doi: 10.1002/art.38298
60. Reilly CM, Gilkeson GS. Use of Genetic Knockouts to Modulate Disease Expression in a Murine Model of Lupus, MRL/lpr Mice. *Immunol Res* (2002) 25:143–53. doi: 10.1385/IR:25:2:143
61. Scalapino KJ, Tang Q, Bluestone JA, Bonyhadi ML, Daikh DI. Suppression of Disease in New Zealand Black/New Zealand White Lupus-Prone Mice by Adoptive Transfer of Ex Vivo Expanded Regulatory T Cells. *J Immunol* (2006) 177:1451–9. doi: 10.4049/jimmunol.177.3.1451
62. Grimaldi CM. Sex and Systemic Lupus Erythematosus: The Role of the Sex Hormones Estrogen and Prolactin on the Regulation of Autoreactive B Cells. *Curr Opin Rheumatol* (2006) 18:456–61. doi: 10.1097/01.bor.0000240354.37927.dd
63. Stannard JN, Kahlenberg JM. Cutaneous Lupus Erythematosus: Updates on Pathogenesis and Associations With Systemic Lupus. *Curr Opin Rheumatol* (2016) 28:453–9. doi: 10.1097/BOR.0000000000000308
64. Jara LJ, Medina G, Saavedra MA, Vera-Lastra O, Navarro C. Prolactin and Autoimmunity. *Clin Rev Allergy Immunol* (2011) 40:50–9. doi: 10.1007/s12016-009-8185-3
65. Nelson P, Rylance P, Roden D, Trela M, Tugnet N. Viruses as Potential Pathogenic Agents in Systemic Lupus Erythematosus. *Lupus* (2014) 23:596–605. doi: 10.1177/0961203314531637
66. Chen M, Aosai F, Norose K, Mun HS, Ishikura H, Hirose S, et al. Toxoplasma Gondii Infection Inhibits the Development of Lupus-Like Syndrome in Autoimmune (New Zealand Black X New Zealand White) F1 Mice. *Int Immunol* (2004) 16:937–46. doi: 10.1093/intimm/dxh095
67. Ram M, Anaya JM, Barzilai O, Izhaky D, Porat Katz BS, Blank M, et al. The Putative Protective Role of Hepatitis B Virus (HBV) Infection From Autoimmune Disorders. *Autoimmun Rev* (2008) 7:621–5. doi: 10.1016/j.autrev.2008.06.008
68. Nockher WA, Wigand R, Schoeppe W, Scherberich JE. Elevated Levels of Soluble CD14 in Serum of Patients With Systemic Lupus Erythematosus. *Clin Exp Immunol* (1994) 96:15–9. doi: 10.1111/j.1365-2249.1994.tb06222.x
69. Shi L, Zhang Z, Yu AM, Wang W, Wei Z, Akhter E, et al. The SLE Transcriptome Exhibits Evidence of Chronic Endotoxin Exposure and Has Widespread Dysregulation of Non-Coding and Coding RNAs. *PloS One* (2014) 9:e93846. doi: 10.1371/journal.pone.0093846
70. Belkaid Y, Hand TW. Role of the Microbiota in Immunity and Inflammation. *Cell* (2014) 157:121–41. doi: 10.1016/j.cell.2014.03.011
71. Hevia A, Milani C, López P, Cuervo A, Arboleya S, Duranti S, et al. Intestinal Dysbiosis Associated With Systemic Lupus Erythematosus. *mBio* (2014) 5:e01548–14. doi: 10.1128/mBio.01548-14
72. Johnson BM, Gaudreau MC, Gudi R, Brown R, Gilkeson G, Vasu C. Gut Microbiota Differently Contributes to Intestinal Immune Phenotype and Systemic Autoimmune Progression in Female and Male Lupus-Prone Mice. *J Autoimmun* (2020) 108:102420. doi: 10.1016/j.jaut.2020.102420
73. Belot A, Kasher PR, Trotter EW, Foray AP, Debaud AL, Rice GI, et al. Protein Kinase Cδ Deficiency Causes Mendelian Systemic Lupus Erythematosus With B Cell-Defective Apoptosis and Hyperproliferation. *Arthritis Rheum* (2013) 65:2161–71. doi: 10.1002/art.38008
74. Al-Mayouf SM, Sunker A, Abdwani R, Abrawi SA, Almurshedi F, Alhashmi N, et al. Loss-Of-Function Variant in DNASE1L3 Causes a Familial Form of Systemic Lupus Erythematosus. *Nat Genet* (2011) 43:1186–8. doi: 10.1038/ng.975
75. Shi G, Abbott KN, Wu W, Salter RD, Keyel PA. Dnase1L3 Regulates Inflammasome-Dependent Cytokine Secretion. *Front Immunol* (2017) 8. doi: 10.3389/fimmu.2017.00522
76. Hardy CJ, Palmer BP, Muir KR, Sutton AJ, Powell RJ. Smoking History, Alcohol Consumption, and Systemic Lupus Erythematosus: A Case-Control Study. *Ann Rheumatic Dis* (1998) 57:451. doi: 10.1136/ard.57.8.451
77. Casciola-Rosen L, Rosen A. Ultraviolet Light-Induced Keratinocyte Apoptosis: A Potential Mechanism for the Induction of Skin Lesions and Autoantibody Production in LE. *Lupus* (1997) 6:175–80. doi: 10.1177/096120339700600213
78. Pawar RD, Ramanjaneyulu A, Kulkarni OP, Lech M, Segerer S, Anders HJ. Inhibition of Toll-Like Receptor-7 (TLR-7) or TLR-7 Plus TLR-9 Attenuates Glomerulonephritis and Lung Injury in Experimental Lupus. *J Am Soc Nephrol* (2007) 18:1721–31. doi: 10.1681/ASN.2006101162

79. Papadimitraki ED, Choulaki C, Koutala E, Bertsias G, Tsatsanis C, Gergianaki I, et al. Expansion of Toll-Like Receptor 9-Expressing B Cells in Active Systemic Lupus Erythematosus: Implications for the Induction and Maintenance of the Autoimmune Process. *Arthritis Rheum* (2006) 54:3601–11. doi: 10.1002/art.22197
80. Christensen SR, Kashgarian M, Alexopoulou L, Flavell RA, Akira S, Shlomchik MJ. Toll-Like Receptor 9 Controls Anti-DNA Autoantibody Production in Murine Lupus. *J Exp Med* (2005) 202:321–31. doi: 10.1084/jem.20050338
81. Munroe ME, Lu R, Zhao YD, Fife DA, Robertson JM, Guthridge JM, et al. Altered Type II Interferon Precedes Autoantibody Accrual and Elevated Type I Interferon Activity Prior to Systemic Lupus Erythematosus Classification. *Ann Rheum Dis* (2016) 75:2014–21. doi: 10.1136/annrheumdis-2015-208140
82. Crow YJ, Manel N. Aicardi–Goutières Syndrome and the Type I Interferonopathies. *Nat Rev Immunol* (2015) 15:429–40. doi: 10.1038/nri3850
83. Talaat RM, Mohamed SF, Bassyouni IH, Raouf AA. Th1/Th2/Th17/Treg Cytokine Imbalance in Systemic Lupus Erythematosus (SLE) Patients: Correlation With Disease Activity. *Cytokine* (2015) 72:146–53. doi: 10.1016/j.cyto.2014.12.027
84. de la Rosa M, Rutz S, Dorninger H, Scheffold A. Interleukin-2 Is Essential for CD4+CD25+ Regulatory T Cell Function. *Eur J Immunol* (2004) 34:2480–8. doi: 10.1002/eji.200425274
85. Laurence A, Tato CM, Davidson TS, Kanno Y, Chen Z, Yao Z, et al. Interleukin-2 Signaling via STAT5 Constrains T Helper 17 Cell Generation. *Immunity* (2007) 26:371–81. doi: 10.1016/j.immuni.2007.02.009
86. Duan JH, Jiang Y, Mu H, Tang ZQ. Expression of BAFF and BR3 in Patients With Systemic Lupus Erythematosus. *Braz J Med Biol Res* (2016) 49:e4853. doi: 10.1590/1414-431X20154853
87. Pers JO, Daridon C, Devauchelle V, Jousse S, Saraux A, Jamin C, et al. BAFF Overexpression Is Associated With Autoantibody Production in Autoimmune Diseases. *Ann N Y Acad Sci* (2005) 1050:34–9. doi: 10.1196/annals.1313.004
88. Holmdahl R, Malmström V, Burkhardt H. Autoimmune Priming, Tissue Attack and Chronic Inflammation - The Three Stages of Rheumatoid Arthritis. *Eur J Immunol* (2014) 44:1593–9. doi: 10.1002/eji.201444486
89. Paul V. Lehmann TF, Alexander Miller EES. Spreading of T-Cell Autoimmunity to Cryptic Determinants of an Autoantigen. *Nature* (1992) 358:155–7. doi: 10.1038/358155a0
90. Vanderlugt CL, Miller SD. Epitope Spreading in Immune-Mediated Diseases: Implications for Immunotherapy. *Nat Rev Immunol* (2002) 2:85–95. doi: 10.1038/nri724
91. Fujinami RS, von Herrath MG, Christen U, Whitton JL. Molecular Mimicry, Bystander Activation, or Viral Persistence: Infections and Autoimmune Disease. *Clin Microbiol Rev* (2006) 19:80–94. doi: 10.1128/CMR.19.1.80-94.2006
92. Yang M, Klocke K, Hernandez CM, Xu B, Gjertsson I, Wing K, et al. Regulatory T Cells Control Epitope Spreading in Autoimmune Arthritis Independent of Cytotoxic T-Lymphocyte Antigen-4. *Immunology* (2018) 155:446–57. doi: 10.1111/imm.12983
93. 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
94. Sinai P, Dozmorov IM, Song R, Schwartzberg PL, Wakeland EK, Wülfing C. T/B-Cell Interactions Are More Transient in Response to Weak Stimuli in SLE-Prone Mice. *Eur J Immunol* (2014) 44:3522–31. doi: 10.1002/eji.201444602
95. Woods M, Zou Y-R, Davidson A. Defects in Germinal Center Selection in SLE. *Front Immunol* (2015) 6:425–5. doi: 10.3389/fimmu.2015.00425
96. Morimoto S, Nakano S, Watanabe T, Tamayama Y, Mitsuo A, Nakiri Y, et al. Expression of B-Cell Activating Factor of the Tumour Necrosis Factor Family (BAFF) in T Cells in Active Systemic Lupus Erythematosus: The Role of BAFF in T Cell-Dependent B Cell Pathogenic Autoantibody Production. *Rheumatology* (2007) 46:1083–6. doi: 10.1093/rheumatology/kem097
97. Bradley SJ, Suarez-Fueyo A, Moss DR, Kyttaris VC, Tsokos GC. T Cell Transcriptomes Describe Patient Subtypes in Systemic Lupus Erythematosus. *PloS One* (2015) 10:e0141171–e0141171. doi: 10.1371/journal.pone.0141171
98. Wing K, Sakaguchi S. Regulatory T Cells Exert Checks and Balances on Self Tolerance and Autoimmunity. *Nat Immunol* (2010) 11:7–13. doi: 10.1038/ni.1818
99. Humrich JY, Morbach H, Undeutsch R, Enghard P, Rosenberger S, Weigert O, et al. Homeostatic Imbalance of Regulatory and Effector T Cells Due to IL-2 Deprivation Amplifies Murine Lupus. *PNAS* (2010) 107:204–9. doi: 10.1073/pnas.0903158107
100. Miyara M, Amoura Z, Parizot C, Badoual C, Dorgham K, Trad S, et al. Global Natural Regulatory T Cell Depletion in Active Systemic Lupus Erythematosus. *J Immunol* (2005) 175:8392–400. doi: 10.4049/jimmunol.175.12.8392
101. Crispin JC, Martínez A, Alcocer-Varela J. Quantification of Regulatory T Cells in Patients With Systemic Lupus Erythematosus. *J Autoimmun* (2003) 21:273–6. doi: 10.1016/S0896-8411(03)00121-5
102. Rowland SL, Riggs JM, Gilfillan S, Bugatti M, Vermi W, Kolbeck R, et al. Early, Transient Depletion of Plasmacytoid Dendritic Cells Ameliorates Autoimmunity in a Lupus Model. *J Exp Med* (2014) 211:1977–91. doi: 10.1084/jem.20132620
103. Davison LM, Jørgensen TN. Sialic Acid-Binding Immunoglobulin-Type Lectin H-Positive Plasmacytoid Dendritic Cells Drive Spontaneous Lupus-Like Disease Development in B6.Nba2 Mice. *Arthritis Rheumatol* (2015) 67:1012–22. doi: 10.1002/art.38989
104. Blomberg S, Eloranta ML, Cederblad B, Nordlin K, Alm GV, Rönnblom L. Presence of Cutaneous Interferon-Alpha Producing Cells in Patients With Systemic Lupus Erythematosus. *Lupus* (2001) 10:484–90. doi: 10.1191/096120301678416042
105. Farkas L, Beiske K, Lund-Johansen F, Brandtzaeg P, Jahnsen FL. Plasmacytoid Dendritic Cells (Natural Interferon- Alpha/Beta-Producing Cells) Accumulate in Cutaneous Lupus Erythematosus Lesions. *Am J Pathol* (2001) 159:237–43. doi: 10.1016/S0002-9440(10)61689-6
106. Blanco P, Palucka AK, Gill M, Pascual V, Banchereau J. Induction of Dendritic Cell Differentiation by IFN-α in Systemic Lupus Erythematosus. *Science* (2001) 294:1540. doi: 10.1126/science.1064890
107. Tawbi HA. *NCT03816345: Nivolumab in Treating Patients With Autoimmune Disorders or Advanced, Metastatic, or Unresectable Cancer*. (2019). ClinicalTrials.gov.
108. Smith G. *NCT03866317: A Study to Assess the Safety and Efficacy of Secukinumab in Alleviating Symptoms of Discoid Lupus Erythematosus*. (2020), ClinicalTrials.gov.
109. Serra P, Santamaria P. Nanoparticle-Based Approaches to Immune Tolerance for the Treatment of Autoimmune Diseases. *Eur J Immunol* (2018) 48:751–6. doi: 10.1002/eji.201747059
110. Tsai S, Shameli A, Yamanouchi J, Clemente-Casares X, Wang J, Serra P, et al. Reversal of Autoimmunity by Boosting Memory-Like Autoregulatory T Cells. *Immunity* (2010) 32:568–80. doi: 10.1016/j.immuni.2010.03.015
111. Clemente-Casares X, Blanco J, Ambalavanan P, Yamanouchi J, Singha S, Fandos C, et al. Expanding Antigen-Specific Regulatory Networks to Treat Autoimmunity. *Nature* (2016) 530:434–40. doi: 10.1038/nature16962
112. Atif M, Conti F, Gorochov G, Oo YH, Miyara M. Regulatory T Cells in Solid Organ Transplantation. *Clin Transl Immunol* (2020) 9. doi: 10.1002/cti2.1099
113. Weigert O, von Spee C, Undeutsch R, Kloke L, Humrich JY, Riemekasten G. CD4+Foxp3+ Regulatory T Cells Prolong Drug-Induced Disease Remission in (NZBxNZW) F1 Lupus Mice. *Arthritis Res Ther* (2013) 15:R35. doi: 10.1186/ar4188
114. Scottà C, Fanelli G, Hoong SJ, Romano M, Lamperti EN, Sukthankar M, et al. Impact of Immunosuppressive Drugs on the Therapeutic Efficacy of Ex Vivo Expanded Human Regulatory T Cells. *1* (2016) 101:91–100.
115. Dall'Era M, Pauli ML, Remedios K, Taravati K, Sandova PM, Putnam AL, et al. Adoptive Treg Cell Therapy in a Patient With Systemic Lupus Erythematosus. *Arthritis Rheumatol* (2019) 71:431–40. doi: 10.1002/art.40737
116. Zhou L, Chong MM, Littman DR. Plasticity of CD4+ T Cell Lineage Differentiation. *Immunity* (2009) 30:646–55. doi: 10.1016/j.immuni.2009.05.001
117. Alexander T, Thiel A, Rosen O, Massenkeil G, Sattler A, Kohler S, et al. Depletion of Autoreactive Immunologic Memory Followed by Autologous Hematopoietic Stem Cell Transplantation in Patients With Refractory SLE Induces Long-Term Remission Through De Novo Generation of a Juvenile

and Tolerant Immune System. *Blood* (2009) 113:214–23. doi: 10.1182/blood-2008-07-168286
118. Zhang L, Bertucci AM, Ramsey-Goldman R, Burt RK, Datta SK. Regulatory T Cell (Treg) Subsets Return in Patients With Refractory Lupus Following Stem Cell Transplantation, and TGF-Beta-Producing CD8+ Treg Cells Are Associated With Immunological Remission of Lupus. *J Immunol (Baltimore Md.* (2009) 1950) 183:6346–58. doi: 10.4049/jimmunol.0901773
119. Wang D, Zhang H, Liang J, Li X, Feng X, Wang H, et al. Allogeneic Mesenchymal Stem Cell Transplantation in Severe and Refractory Systemic Lupus Erythematosus: 4 Years of Experience. *Cell Transplant* (2013) 22:2267–77. doi: 10.3727/096368911X582769c
120. Dauphinée MJ, Kipper SB, Wofsy D, Talal N. Interleukin 2 Deficiency is a Common Feature of Autoimmune Mice. *J Immunol* (1981) 127:2483–7.
121. Linker-Israeli M, Bakke AC, Kitridou RC, Gendler S, Gillis S, Horwitz DA. Defective Production of Interleukin 1 and Interleukin 2 in Patients With Systemic Lupus Erythematosus (SLE). *J Immunol* (1983) 130:2651–5.
122. 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
123. Fontenot JD, Rasmussen JP, Gavin MA, Rudensky AY. A Function for Interleukin 2 in Foxp3-Expressing Regulatory T Cells. *Nat Immunol* (2005) 6:1142–51. doi: 10.1038/ni1263
124. Gómez-Martín D, Díaz-Zamudio M, Crispín JC, Alcocer-Varela J. Interleukin 2 and Systemic Lupus Erythematosus: Beyond the Transcriptional Regulatory Net Abnormalities. *Autoimmun Rev* (2009) 9:34–9. doi: 10.1016/j.autrev.2009.02.035
125. Crispín JC, Tsokos GC. IL-17 in Systemic Lupus Erythematosus. *J Biomed Biotechnol* (2010). doi: 10.1155/2010/943254
126. Mizui M, Koga T, Lieberman LA, Beltran J, Yoshida N, Johnson MC, et al. IL-2 Protects Lupus-Prone Mice From Multiple End-Organ Damage by Limiting CD4–CD8– IL-17–Producing T Cells. *J Immunol* (2014) 193:2168–77. doi: 10.4049/jimmunol.1400977
127. Meropol NJ, Porter M, Blumenson LE, Lindemann MJ, Perez RP, Vaickus L, et al. Daily Subcutaneous Injection of Low-Dose Interleukin 2 Expands Natural Killer Cells In Vivo Without Significant Toxicity. *Clin Cancer Res* (1996) 2:669–77.
128. von Spee-Mayer C, Siegert E, Abdirama D, Rose A, Klaus A, Alexander T, et al. Low-Dose Interleukin-2 Selectively Corrects Regulatory T Cell Defects in Patients With Systemic Lupus Erythematosus. *Ann Rheum Dis* (2016) 75 (7):1407–15. doi: 10.1136/annrheumdis-2015-207776
129. McHugh MD, Park J, Uhrich R, Gao W, Horwitz DA, Fahmy TM. Paracrine Co-Delivery of TGF-β and IL-2 Using CD4-Targeted Nanoparticles for Induction and Maintenance of Regulatory T Cells. *Biomaterials* (2015) 59:172–81. doi: 10.1016/j.biomaterials.2015.04.003
130. Siddhanti S, Fanton C, Dixit N, Lu L, Chindalore V, Levin R, et al. THU0054 NKTR-358, a Novel IL-2 Conjugate, Stimulates High Levels of Regulatory T Cells in Patients With Systemic Lupus Erythematosus. *Ann Rheum Dis* (2020) 79. doi: 10.1136/annrheumdis-2020-eular.3165
131. Hawiger D, Inaba K, Dorsett Y, Guo M, Mahnke K, Rivera M, et al. Dendritic Cells Induce Peripheral T Cell Unresponsiveness Under Steady State Conditions in Vivo. *J Exp Med* (2001) 194:769–80. doi: 10.1084/jem.194.6.769
132. Mansilla MJ, Sellès-Moreno C, Fàbregas-Puig S, Amoedo J, Navarro-Barriuso J, Teniente-Serra A, et al. Beneficial Effect of Tolerogenic Dendritic Cells Pulsed With MOG Autoantigen in Experimental Autoimmune Encephalomyelitis. *CNS Neurosci Ther* (2014) 21:222–30. doi: 10.1111/cns.12342
133. Van Brussel I, Lee WP, Rombouts M, Nuyts AH, Heylen M, De Winter BY, et al. Tolerogenic Dendritic Cell Vaccines to Treat Autoimmune Diseases: Can the Unattainable Dream Turn Into Reality? *Autoimmun Rev* (2014) 13:138–50. doi: 10.1016/j.autrev.2013.09.008
134. Rosenzwajg M, Lorenzon R, Cacoub P, Pham HP, Pitoiset F, El Soufi K, et al. Immunological and Clinical Effects of Low-Dose Interleukin-2 Across 11 Autoimmune Diseases in a Single, Open Clinical Trial. *Ann Rheum Dis* (2019) 78(2):209–17. doi: 10.1136/annrheumdis-2018-214229
135. Willekens B, Presas-Rodríguez S, Mansilla MJ, Derdelinckx J, Lee W-P, Nijs G, et al. Tolerogenic Dendritic Cell-Based Treatment for Multiple Sclerosis (MS): A Harmonised Study Protocol for Two Phase I Clinical Trials Comparing Intradermal and Intranodal Cell Administration. *BMJ Open* (2019) 9:e030309. doi: 10.1136/bmjopen-2019-030309
136. Benham H, Nel HJ, Law SC, Mehdi AM, Street S, Ramnoruth N, et al. Citrullinated Peptide Dendritic Cell Immunotherapy in HLA Risk Genotype-Positive Rheumatoid Arthritis Patients. *Sci Transl Med* (2015) 7:290ra87. doi: 10.1126/scitranslmed.aaa9301
137. Bell GM, Anderson AE, Diboll J, Reece R, Eltherington O, Harry RA, et al. Autologous Tolerogenic Dendritic Cells for Rheumatoid and Inflammatory Arthritis. *Ann Rheumatic Dis* (2017) 76:227–34. doi: 10.1136/annrheumdis-2015-208456
138. Funes SC, Rios M, Gomez-Santander F, Fernandez-Fierro A, Altamirano-Lagos MJ, Rivera-Perez D, et al. Tolerogenic Dendritic Cell Transfer Ameliorates Systemic Lupus Erythematosus in Mice. *Immunology* (2019) 158:322–39. doi: 10.1111/imm.13119
139. Obreque J, Vega F, Torres A, Cuitino L, Mackern-Oberti JP, Viviani P, et al. Autologous Tolerogenic Dendritic Cells Derived From Monocytes of Systemic Lupus Erythematosus Patients and Healthy Donors Show a Stable and Immunosuppressive Phenotype. *Immunology* (2017) 152:648–59. doi: 10.1111/imm.12806
140. Look M, Stern E, Wang QA, DiPlacido LD, Kashgarian M, Craft J, et al. Nanogel-Based Delivery of Mycophenolic Acid Ameliorates Systemic Lupus Erythematosus in Mice. *J Clin Invest* (2013) 123:1741–9. doi: 10.1172/JCI65907
141. Look M, Saltzman WM, Craft J, Fahmy TM. The Nanomaterial-Dependent Modulation of Dendritic Cells and Its Potential Influence on Therapeutic Immunosuppression in Lupus. *Biomaterials* (2014) 35:1089–95. doi: 10.1016/j.biomaterials.2013.10.046
142. Monneaux F, Briand J-P, Muller S. B and T Cell Immune Response to Small Nuclear Ribonucleoprotein Particles in Lupus Mice: Autoreactive CD4+ T Cells Recognize a T Cell Epitope Located Within the RNP80 Motif of the 70K Protein. *Eur J Immunol* (2000) 30:2191–200. doi: 10.1002/1521-4141(2000)30:8<2191::AID-IMMU2191>3.0.CO;2-R
143. Monneaux F, Dumortier H, Steiner G, Briand J-P, Muller S. Murine Models of Systemic Lupus Erythematosus: B and T Cell Responses to Spliceosomal Ribonucleoproteins in MRL/Faslpr and (NZB × NZW)F1 Lupus Mice. *Int Immunol* (2001) 13:1155–63. doi: 10.1093/intimm/13.9.1155
144. Wang F, Tasset I, Cuervo AM, Muller S. In Vivo Remodeling of Altered Autophagy-Lysosomal Pathway by a Phosphopeptide in Lupus. *Cells* (2020) 9. doi: 10.3390/cells9102328
145. Muller S, Monneaux F, Schall N, Rashkov RK, Oparanov BA, Wiesel P, et al. Spliceosomal Peptide P140 for Immunotherapy of Systemic Lupus Erythematosus: Results of an Early Phase II Clinical Trial. *Arthritis Rheumatism* (2008) 58:3873–83. doi: 10.1002/art.24027
146. Zimmer R, Scherbarth HR, Rillo OL, Gomez-Reino JJ, Muller S. Lupuzor/P140 Peptide in Patients With Systemic Lupus Erythematosus: A Randomised, Double-Blind, Placebo-Controlled Phase IIb Clinical Trial. *Ann Rheumatic Dis* (2013) 72:1830. doi: 10.1136/annrheumdis-2012-202460
147. *Add-On Lupuzor Fails Primary Goal But Shows Some Positive Results in Phase 3 Trial for SLE.*
148. Alhadj Ali M, Liu Y-F, Arif S, Tatovic D, Shariff H, Gibson VB, et al. Metabolic and Immune Effects of Immunotherapy With Proinsulin Peptide in Human New-Onset Type 1 Diabetes. *Sci Transl Med* (2017) 9. doi: 10.1126/scitranslmed.aaf7779
149. Santambrogio L, Sato AK, Fischer FR, Dorf ME, Stern LJ. Abundant Empty Class II MHC Molecules on the Surface of Immature Dendritic Cells. *Proc Natl Acad Sci USA* (1999) 96:15050–5. doi: 10.1073/pnas.96.26.15050
150. Santambrogio L, Strominger JL. The Ins and Outs of MHC Class II Proteins in Dendritic Cells. *Immunity* (2006) 25:857–9. doi: 10.1016/j.immuni.2006.11.007
151. Burton BR, Britton GJ, Fang H, Verhagen J, Smithers B, Sabatos-Peyton CA, et al. Sequential Transcriptional Changes Dictate Safe and Effective Antigen-Specific Immunotherapy. *Nat Commun* (2014) 5:4741. doi: 10.1038/ncomms5741
152. Bevington SL, Ng STH, Britton GJ, Keane P, Wraith DC, Cockerill PN. Chromatin Priming Renders T Cell Tolerance-Associated Genes Sensitive to Activation Below the Signaling Threshold for Immune Response Genes. *Cell Rep* (2020) 31:107748. doi: 10.1016/j.celrep.2020.107748

153. Chihara N, Madi A, Kondo T, Zhang H, Acharya N, Singer M, et al. Induction and Transcriptional Regulation of the Co-Inhibitory Gene Module in T Cells. *Nature* (2018) 558:454–9. doi: 10.1038/s41586-018-0206-z
154. Capini C, Jaturanpinyo M, Chang H-I, Mutalik S, McNally A, Street S, et al. Antigen-Specific Suppression of Inflammatory Arthritis Using Liposomes. *J Immunol* (2009) 182:3556–65. doi: 10.4049/jimmunol.0802972
155. Galea R, Nel HJ, Talekar M, Liu X, Ooi JD, Huynh M, et al. PD-L1– and Calcitriol-Dependent Liposomal Antigen-Specific Regulation of Systemic Inflammatory Autoimmune Disease. *JCI Insight* (2019) 4:e126025. doi: 10.1172/jci.insight.126025
156. Maldonado RA, LaMothe RA, Ferrari JD, Zhang A-H, Rossi RJ, Kolte PN, et al. Polymeric Synthetic Nanoparticles for the Induction of Antigen-Specific Immunological Tolerance. *PNAS* (2015) 112:E156–65. doi: 10.1073/pnas.1408686111
157. Gutiérrez-Vázquez C, Quintana FJ. Regulation of the Immune Response by the Aryl Hydrocarbon Receptor. *Immunity* (2018) 48:19–33. doi: 10.1016/j.immuni.2017.12.012
158. Hlavaty KA, McCarthy DP, Saito E, Yap WT, Miller SD, Shea LD. Tolerance Induction Using Nanoparticles Bearing HY Peptides in Bone Marrow Transplantation. *Biomaterials* (2016) 76:1–10. doi: 10.1016/j.biomaterials.2015.10.041
159. Getts DR, Martin AJ, McCarthy DP, Terry RL, Hunter ZN, Yap WT, et al. Microparticles Bearing Encephalitogenic Peptides Induce T-Cell Tolerance and Ameliorate Experimental Autoimmune Encephalomyelitis. *Nat Biotechnol* (2012) 30:1217–24. doi: 10.1038/nbt.2434
160. Getts DR, Shea LD, Miller SD, King NJC. Harnessing Nanoparticles for Immune Modulation. *Trends Immunol* (2015) 36:419–27. doi: 10.1016/j.it.2015.05.007
161. Pujol-Autonell I, Serracant-Prat A, Cano-Sarabia M, Ampudia RM, Rodriguez-Fernandez S, Sanchez A, et al. Use of Autoantigen-Loaded Phosphatidylserine-Liposomes to Arrest Autoimmunity in Type 1 Diabetes. *PloS One* (2015) 10:e0127057. doi: 10.1371/journal.pone.0127057
162. Roberts RA, Eitas TK, Byrne JD, Johnson BM, Short PJ, McKinnon KP, et al. Towards Programming Immune Tolerance Through Geometric Manipulation of Phosphatidylserine. *Biomaterials* (2015) 72:1–10. doi: 10.1016/j.biomaterials.2015.08.040
163. Singha S, Shao K, Yang Y, Clemente-Casares X, Solé P, Clemente A, et al. Peptide–MHC-Based Nanomedicines for Autoimmunity Function as T-Cell Receptor Microclustering Devices. *Nat Nanotechnol* (2017) 12:701–10. doi: 10.1038/nnano.2017.56
164. Anderton SM, Wraith DC. Hierarchy in the Ability of T Cell Epitopes to Induce Peripheral Tolerance to Antigens From Myelin. *Eur J Immunol* (1998) 28:1251–61. doi: 10.1002/(SICI)1521-4141(199804)28:04<1251::AID-IMMU1251>3.0.CO;2-O
165. Bergot A-S, Buckle I, Cikaluru S, Naranjo JL, Wright CM, Zheng G, et al. Regulatory T Cells Induced by Single-Peptide Liposome Immunotherapy Suppress Islet-Specific T Cell Responses to Multiple Antigens and Protect From Autoimmune Diabetes. *J Immunol* (2020), ji1901128. doi: 10.4049/jimmunol.1901128
166. Graham RR, Ortmann WA, Langefeld CD, Jawaheer D, Selby SA, Rodine PR, et al. Visualizing Human Leukocyte Antigen Class II Risk Haplotypes in Human Systemic Lupus Erythematosus. *Am J Hum Genet* (2002) 71:543–53. doi: 10.1086/342290
167. Deshmukh US, Sim DL, Dai C, Kannapell CJ, Gaskin F, Rajagopalan G, et al. HLA-DR3 Restricted T Cell Epitope Mimicry in Induction of Autoimmune Response to Lupus-Associated Antigen SmD. *J Autoimmun* (2011) 37:254–62. doi: 10.1016/j.jaut.2011.07.002
168. Kaliyaperumal A, Mohan C, Wu W, Datta SK. Nucleosomal Peptide Epitopes for Nephritis-Inducing T Helper Cells of Murine Lupus. *J Exp Med* (1996) 183:2459–69. doi: 10.1084/jem.183.6.2459
169. Zhang L, Bertucci AM, Ramsey-Goldman R, Harsha-Strong ER, Burt RK, Datta SK. Major Pathogenic Steps in Human Lupus can be Effectively Suppressed by Nucleosomal Histone Peptide Epitope-Induced Regulatory Immunity. *Clin Immunol* (2013) 149:365–78. doi: 10.1016/j.clim.2013.08.008
170. Stemmer C, Briand J-P, Muller S. Mapping of Linear Histone Regions Exposed at the Surface of the Nucleosome in Solution11edited by T. *Richmond J Mol Biol* (1997) 273:52–60. doi: 10.1006/jmbi.1997.1270
171. Muller S. BD, Thiry M, Van Regenmortel MHV. Reactivity of Autoantibodies in Systemic Lupus Etythematosus With Synthetic Core Histone Peptides. *Int Arch Allergy Immunol* (1989) 89:288–96. doi: 10.1159/000234962
172. Kang HK, Liu M, Datta SK. Low-Dose Peptide Tolerance Therapy of Lupus Generates Plasmacytoid Dendritic Cells That Cause Expansion of Autoantigen-Specific Regulatory T Cells and Contraction of Inflammatory Th17 Cells. *J Immunol* (2007) 178:7849–58. doi: 10.4049/jimmunol.178.12.7849
173. Kang HK, Chiang MY, Liu M, Ecklund D, Datta SK. The Histone Peptide H4 71-94 Alone Is More Effective Than a Cocktail of Peptide Epitopes in Controlling Lupus: Immunoregulatory Mechanisms. *J Clin Immunol* (2011) 31:379–94. doi: 10.1007/s10875-010-9504-4
174. Sabbatini A, Bombardieri S, Migliorini P. Autoantibodies From Patients With Systemic Lupus Erythematosus Bind a Shared Sequence of SmD and Epstein-Barr Virus-Encoded Nuclear Antigen EBNA I. *Eur J Immunol* (1993) 23:1146–52. doi: 10.1002/eji.1830230525
175. Nel HJ, Malmström V, Wraith DC, Thomas R. Autoantigens in Rheumatoid Arthritis and the Potential for Antigen-Specific Tolerising Immunotherapy. *Lancet Rheumatol* (2020), e712–23. doi: 10.1016/S2665-9913(20)30344-1
176. Gunawan M, Her Z, Liu M, Tan SY, Chan XY, Tan WWS, et al. A Novel Human Systemic Lupus Erythematosus Model in Humanised Mice. *Sci Rep* (2017) 7:16642. doi: 10.1038/s41598-017-16999-7
177. Mihaylova N, Chipinski P, Bradyanova S, Velikova T, Ivanova-Todorova E, Chausheva S, et al. Suppression of Autoreactive T and B Lymphocytes by Anti-Annexin A1 Antibody in a Humanized NSG Murine Model of Systemic Lupus Erythematosus. *Clin Exp Immunol* (2020) 199:278–93. doi: 10.1111/cei.13399
178. Maria Dall'Era AH, Bluestone J, Rosenblum M, Wofsy D. *NCT02428309: Autologous Polyclonal Tregs for Lupus*. (2015), ClinicalTrials.gov.
179. Deshmukh US, Gaskin F, Lewis JE, Kannapell CC, Fu SM. Mechanisms of Autoantibody Diversification to SLE-Related Autoantigens. *Ann NY Acad Sci* (2003) 987:91–8. doi: 10.1111/j.1749-6632.2003.tb06036.x
180. Riemekasten G, Langnickel D, Ebling FM, Karpouzas G, Kalsi J, Herberth G, et al. Identification and Characterization of SmD183-119-Reactive T Cells That Provide T Cell Help for Pathogenic Anti-Double-Stranded DNA Antibodies. *Arthritis Rheum* (2003) 48:475–85. doi: 10.1002/art.10762
181. James JA, Gross T, Scofield RH, Harley JB. Immunoglobulin Epitope Spreading and Autoimmune Disease After Peptide Immunization: Sm B/B'-Derived PPPGMRPP and PPPGIRGP Induce Spliceosome Autoimmunity. *J Exp Med* (1995) 181:453–61. doi: 10.1084/jem.181.2.453
182. Reynolds P, Gordon TP, Purcell AW, Jackson DC, McCluskey J. Hierarchical Self-Tolerance to T Cell Determinants Within the Ubiquitous Nuclear Self-Antigen La (SS-B) Permits Induction of Systemic Autoimmunity in Normal Mice. *J Exp Med* (1996) 184:1857–70. doi: 10.1084/jem.184.5.1857
183. Dumortier H, Monneaux F, Jahn-Schmid B, Briand J-P, Skriner K, Cohen PL, et al. B and T Cell Responses to the Spliceosomal Heterogeneous Nuclear Ribonucleoproteins A2 and B1 in Normal and Lupus Mice. *J Immunol* (2000) 165:2297. doi: 10.4049/jimmunol.165.4.2297
184. Mohan C, Adams S, Stanik V, Datta SK. Nucleosome: A Major Immunogen for Pathogenic Autoantibody-Inducing T Cells of Lupus. *J Exp Med* (1993) 177:1367–81. doi: 10.1084/jem.177.5.1367
185. Ravirajan CT, Muller S, Katz DR, Isenberg DA. Effect of Histone and Histone-RNA Complexes on the Disease Process of Murine Systemic Lupus Erythematosus. *Autoimmunity* (1995) 21:117–22. doi: 10.3109/08916939508993358

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.

frontiers in Immunology

REVIEW
published: 03 September 2021
doi: 10.3389/fimmu.2021.731947

Regulatory T Cells in Autoimmunity and Cancer: A Duplicitous Lifestyle

*Aikaterini Hatzioannou[1], Athina Boumpas[2], Miranta Papadopoulou[2], Iosif Papafragkos[3,4], Athina Varveri[2], Themis Alissafi[2] and Panayotis Verginis[1,2,3,4]**

[1] Institute for Clinical Chemistry and Laboratory Medicine, Faculty of Medicine, Technische Universität Dresden, Dresden, Germany, [2] Center of Clinical, Experimental Surgery & Translational Research, Biomedical Research Foundation of the Academy of Athens, Athens, Greece, [3] Institute of Molecular Biology and Biotechnology (IMBB), Foundation for Research and Technology - Hellas (FORTH), Heraklion, Greece, [4] Laboratory of Immune Regulation and Tolerance, Division of Basic Sciences, Medical School, University of Crete, Heraklion, Greece

Regulatory T (Treg) cells, possess a strategic role in the maintenance of immune homeostasis, and their function has been closely linked to development of diverse pathologies including autoimmunity and cancer. Comprehensive studies in various disease contexts revealed an increased plasticity as a characteristic of Treg cells. Although Treg cell plasticity comes in various flavors, the major categories enclose the loss of Foxp3 expression, which is the master regulator of Treg cell lineage, giving rise to "ex-Treg" cells and the "fragile" Treg cells in which *FOXP3* expression is retained but accompanied by the engagement of an inflammatory program and attenuation of the suppressive activity. Treg cell plasticity possess a tremendous therapeutic potential either by inducing Treg cell de-stabilization to promote anti-tumor immunity, or re-enforcing Treg cell stability to attenuate chronic inflammation. Herein, we review the literature on the Treg cell plasticity with lessons learned in autoimmunity and cancer and discuss challenges and open questions with potential therapeutic implications.

Keywords: regulatory T cell, autoimmune disease, cancer, tolerance, immunotherapy

OPEN ACCESS

Edited by:
Antonio La Cava, University of California, Los Angeles, United States

Reviewed by:
Hye-Jung Kim, Dana–Farber Cancer Institute, United States
Choong-Gu Lee, Korea Institute of Science and Technology (KIST), South Korea

***Correspondence:**
Panayotis Verginis
pverginis@uoc.gr

Specialty section:
This article was submitted to Immunological Tolerance and Regulation, a section of the journal Frontiers in Immunology

Received: *28 June 2021*
Accepted: *18 August 2021*
Published: *03 September 2021*

Citation:
Hatzioannou A, Boumpas A, Papadopoulou M, Papafragkos I, Varveri A, Alissafi T and Verginis P (2021) Regulatory T Cells in Autoimmunity and Cancer: A Duplicitous Lifestyle. Front. Immunol. 12:731947. doi: 10.3389/fimmu.2021.731947

INTRODUCTION

Over the last decades T regulatory (Treg) cells have emerged as a novel regulator of the immune system and several approaches have been proposed for their therapeutic targeting in autoimmune diseases, transplantation and cancer. For example, daily administration of low doses interleukin 2 (IL-2) has been linked to expansion of Treg cells and amelioration of graft-vs-host disease as well as induction of remission in systemic lupus erythematosus (SLE) and type I diabetes. On the other hand, immune checkpoint immunotherapy (ICI) in cancer is based on targeting molecules that are abundantly expressed by Treg cells, such as cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and program cell death protein 1 (PD-1), suggesting that therapeutic efficacy may depend on this powerful suppressive cell subset. Accumulating knowledge however, points to an increased plasticity of the Treg cell compartment expressed with multiple "faces" including loss of suppressive function, expression of inflammatory cytokines and re-programming of their transcription program. Although it remains unclear which factors dictate Treg cell plasticity it is possible that specific microenvironments imprint on Treg cell fate. Therefore, understanding the mechanisms that mediate Treg cell plasticity is of paramount importance and should be considered

during the design of Treg cell therapeutic protocols as well as other treatments that directly or indirectly influence Treg cell homeostasis. In this review we discuss current knowledge on Treg cell plasticity with emphasis in autoimmunity and cancer.

TREG CELL IDENTITY CARD

Treg cells constitute the immunosuppressive subpopulation of $CD4^+$ T cells, representing approximately 5-10% of peripheral $CD4^+$ T cells in blood of healthy individuals (1, 2). They are characterized by the expression of the transcription factor forkhead P3 (FOXP3) (3, 4), a transcription factor instrumental for the development and function of these cells. To this end, individuals, and specifically men, bearing loss-of function mutation in their *FOXP3* gene, have been reported to develop severe systemic multi-organ inflammation and autoimmune disorder, known as immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome (5, 6). Similar to human, murine hemizygous males with an X-linked frame shift mutation in their *Foxp3* gene manifest a scurfy phenotype, characterized by hyperactivation and expansion of autoreactive $CD4^+$ T cells leading to a lethal inflammatory multi-organ failure (3, 7, 8). In accordance, ectopic expression of Foxp3 confers suppressor function on T effector cells proving the importance of this transcription factor as a critical regulator of Treg development and function (3). Specifically, Foxp3 binds to many genes and acts as both a transcriptional activator and repressor regulating the expression of genes encoding nuclear factors that control gene expression and chromatin remodeling (9). The capacity of Foxp3 to both activate and repress transcription is content and partner-dependent. Thus, it acts as an activator when complexed with the transcriptional factors RELA, IKZF2 and KAT5 and as a repressor when complexed with histone methyltransferase EZH2 and transcription factors YY1 and IKZF3 (10). Moreover, Foxp3 facilitates the formation of repressive chromatin in Treg cells upon their activation in response to inflammatory cues (11). For instance Foxp3 represses cyclic nucleotide phosphodiesterase 3B, affecting genes responsible for Treg cell homeostasis and amplifies molecular features of Treg cells, such as anergy and dependence on paracrine IL-2 (12). On the other hand other studies have demonstrated that Foxp3 defines Treg cell identity indirectly by fine-tuning the activity of other major chromatin remodeling TFs such as TCF1 (13). Up to date two subsets of Foxp3-expressing Tregs have been described: those emerging *de novo* in the thymus ("thymic" or tTregs) and those induced in the periphery ("peripheral" or pTregs).

Apart from *FOXP3* expression, Treg cells abundantly express CD25 (IL-2Rα), which is the low-avidity IL-2 receptor and is crucial for the development and the maintenance of Treg cells (14–16). They also express co-inhibitory molecules such as PD-1, CTLA-4, T cell immunoreceptor with Ig and ITIM domains (TIGIT), V-domain Ig suppressor of T cell activation (VISTA), T cell immunoglobulin mucin 3 (TIM-3) and lymphocyte activation gene-3 (LAG-3) as well as co-stimulatory molecules, such as glucocorticoid-induced TNFR-related protein (GITR), 4-1BB (CD137), inducible T cell co-stimulator (ICOS) and OX-40 (CD134). These molecules are responsible for Treg cell suppressive function and/or activation while their manipulation has been closely linked to Treg cell functional instability in diverse disease settings (17).

Multiple mechanisms have been described *via* which Tregs exert their suppressive activity and can be broadly classified into four distinct categories: 1) secretion of immunosuppressive cytokines, 2) cytolysis, 3) metabolic disruption, 4) suppression of dendritic cells (DC) maturation and function. In more details:

Immunosuppressive Cytokine Secretion

Inhibitory cytokines, including IL-10, tumor growth factor β (TGF-β), IL-35, are abundantly secreted by Treg cells, orchestrating their function. For instance, IL-10 production by Treg cells decreases the interferon (IFN)-γ-dependent activation of antigen presenting cells (APCs), suppresses IFN-γ production in $CD8^+$ cells and induces downregulation of major histocompatibility complex (MHC) II and CD86 in tumor-associated macrophages (18, 19). Similarly, IL-10-producing Treg cells control autoimmunity (20, 21). TGF-β secretion by Treg cells exerts a plethora of immunosuppressive effects, including blockade of DC priming and lymphocyte survival, favoring an anti-inflammatory phenotype in macrophages and inhibiting natural killer (NK) cell effector function in the context of both autoimmunity and cancer (22–24). Lastly, IL-35 secretion has been described to induce cell cycle arrest in T cells through the janus kinases (JAK) - signal transducer and activator of transcription proteins (STAT) pathway, thus potentiating inhibition of T cell proliferation in the tumor microenvironment (TME) and suppression of autoimmune diseases, such as colitis (25).

Cytolysis

Cytolysis is a Treg cell suppressive mechanism described mainly in cancer and *in vitro* studies. Targeted cells ($CD4^+$, $CD8^+$ effector T cells, B cells, and NK cells) are driven to apoptosis by Treg cell secreted granzymes in either perforin-dependent or independent manner. Mechanistically, activated Treg cells, through the expression of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), bind to death receptor (1) on target cells leading to apoptotic-mediated cytolysis (26–28). This is the mechanism exploited by tumor-infiltrating Treg cells to trigger apoptosis in NK cells (29), B cells, DC and cytotoxic T cells of the TME (26, 30, 31).

"Metabolic Disruption"

Treg cells have the ability to modulate effector cell function by interfering with cell metabolism in an antigen-non-specific manner. To this end, IL2R-expressing Treg cells consume the surrounding IL-2, negatively affecting CD4+ and CD8+ cell proliferative response (32). In cancer, Treg cells express high levels of CD25, actively consuming IL-2, suppressing the activation and proliferation of effector T cells (33) and promoting their apoptosis (34). Induction of apoptosis in

autoreactive T cells due to Treg cell-induced IL-2 deprivation has also been described in the T cell adoptive transfer model of inflammatory bowel disease (35). Another well-described suppressive Treg cell mechanism is the production of adenosine from the conversion of extracellular adenosine triphosphate (ATP) by the ectonucleotidases CD39 and CD73 expressed on the cell surface of Treg cells. CD39 expression is driven by Foxp3 and its catalytic activity is strongly enhanced by T-cell receptor (TCR) ligation (36). Interestingly, in the TME, apoptotic Treg cells are the source of extracellular ATP, which is subsequently metabolized into adenosine by live Treg cells (37). Adenosine is a metabolite, which suppresses T cell, DC and pro-inflammatory macrophage maturation and function (17, 33, 38, 39). In support, patients with the remitting/relapsing form of multiple sclerosis (5) have strikingly reduced numbers of $CD39^+$ Treg cells in the blood (40).

Suppression of DC Maturation and Function

A major mechanism of Treg cell-mediated immunosuppression is the inhibition of the immunological synapse between effector T cells and APCs, resulting in impaired APC maturation and T cell anergy. Treg cells, through expression of inhibitory receptors (e.g., CTLA-4), engage the co-stimulatory molecules CD80/CD86 on DC with a higher affinity than CD28, impeding DC maturation and function (41, 42). Furthermore, through CTLA-4, Treg cells capture co-stimulatory molecules on DC by the process of transendocytosis (43), while *in vitro* assays have shown that Tregs down-regulate CD80 and CD86 expression in DC in a CTLA-4 and lymphocyte function-associated antigen (LFA)-1 dependent manner, highly blocking or weakening the signaling between APCs and anti-tumor specific T cells (44). Additionally, Treg-CTLA-4 increases Indoleamine-pyrrole 2,3-dioxygenase (IDO) expression in the DC, which lowers the concentration of tryptophan necessary for T effector cells to proliferate (45). In accordance, our group recently demonstrated that $Foxp3^+$ Treg cells potently suppress autoimmune responses *in vivo* through inhibition of the autophagic machinery in DC in a CTLA-4-dependent manner (46). Moreover, Treg cells have been demonstrated to accomplish prolonged interactions with DC in an neuropilin (Nrp)-1/MHC II dependent fashion, a process able to further limit the access of effector T cells (47). Finally, through expression of LAG-3, which is a homolog for CD4, Treg cells have been reported to suppress DC function by LAG-3/MHC II interactions (48).

TREG CELL METABOLISM

Cellular metabolism has emerged as a crucial parameter to influence Treg cell lineage stability, survival, proliferation and function in immune homeostasis but also during pathological situations (23, 33, 45, 49–52). Treg cells exhibit a unique metabolic signature compared with conventional effector T cells. Specifically, meeting of the energy needs of Treg cells suppressive activity, is based on mitochondria metabolism and mainly on elevated levels of fatty acid oxidation (32, 53, 54). As an example, Treg cells are characterized by a metabolic advantage in the nutrient-deprived, lactate-rich, highly hypoxic TME, compared to $CD4^+$ T effector and $CD8^+$ cytotoxic T cells, which rely primarily on anabolism and glycolysis to support their bioenergetic needs (23). Transcriptomic analysis has shown that human intra-tumoral Treg cells upregulate genes related to lipid synthesis, while in tumor mouse models Treg cells display increased fatty acid synthesis (55); in fact, Foxp3 expression in T cells has been shown to induce oxidative phosphorylation and suppress glycolysis in mouse models (33). Overall, metabolic signaling has emerged as a main component in defining Treg cell function and fate. Thus, understanding how the microenvironment affects the metabolic decisions of Treg cells may help in the delineation of pathogenic mechanisms and can pave the way for novel immunotherapeutic approaches.

GENETIC AND EPIGENETIC PROGRAM OF THE *FOXP3* LOCUS

Regulatory Elements of the *Foxp3* Locus

The human *FOXP3* gene is located in the p-arm of the X chromosome and is one of the most intensively studied genes in recent years. The *FOXP3* promoter, positioned in the 1st intron, relies on other cis-regulatory elements. Comparative genomic approaches discovered four conserved non-coding sequences (CNSs) on *Foxp3* locus: 1) regulatory CSN0, located on an intron of the neighbouring gene 5′ of the *Foxp3* locus, 2) intronic enhancer CNS1, located in the 1st intron, along with, 3) CNS2, known as Treg cell-specific demethylated region, 4) CNS3, located directly after exon 1 (56). CNS0 is the most recently discovered regulatory element/super-enhancer, contributing to tTReg cells generation; also regulated by CNS3 (51, 53, 57). On the other hand, CNS1 is redundant for nTreg cell development, while also related to the development of iTreg cells. CNS2 contains highly conserved CpG motifs, known as Treg cell specific demethylated regions (TSDR) that represent the most definitive marker of commitment to the Treg cell lineage (58–61). Importantly, it has been demonstrated that CNS2 deletion affects the stability of *Foxp3* expression during proliferation (56, 57, 62–65).

Transcription Factors Binding to *FOXP3* Regulatory Elements

Several transcription factors have been described to bind either to the *FOXP3* promoter or to the CNS regions to induce or maintain *FOXP3* expression. The FOXO family of transcription factors directly binds to CNS1, CNS3 areas and indirectly regulates Treg cell-specific genes, *SMAD3* and nuclear factor of activated T-cells (*NFAT*) (66, 67). NFAT binds to CNS1 upon TCR-signalling, while SMAD3 after TGF-β binding (65). Furthermore, Stat5, which is activated upon IL-2 signalling, binds to CNS2, protecting Treg cell identity from other cytokine signals and maintaining heritable transcription of *Foxp3* (68).

Epigenetic Regulation

Post-translational mechanisms regulate *Foxp3* expression positively and negatively through methylation, acetylation, phosphorylation, and ubiquitination. Transcription factors cAMP response element-binding protein (CREB)/activating transcription factor (ATF), nuclear factor 'kappa-light-chain-enhancer' of activated B-cells (NF-κB), Ets-1 and the Runx-Foxp3 complex, all Foxp3-inducers, cannot bind to CNS2 without demethylation (56, 59, 69). Environmental factors, such as Vitamin C, induces CNS2 demethylation in Treg cells in a ten-eleven-translocation 2 (Tet-2)-dependent manner (70). Additionally, TSDR demethylation is facilitated by superagonist CD28, high expression of CD45RA or CD39, and IL-2/CD25 (10, 71, 72). Histone modification also contributes to *Foxp3* expression, with trimethylation of H3K4 on the promoter and CNS1 regions being strongly correlated with *Foxp3* expression in fully differentiated Treg cells (73, 74). Opposingly, TDSR methylation destabilizes *Foxp3* expression and impairs Treg cells suppressive activity. Acetylation is also associated with Treg cells stability and function either by Foxp3 or histone acetylation. TGF-surface signalling assists Foxp3 acetylation (75, 76), while methyl-CpG binding protein two and galectin-9/CD44 pathway promote respectively CNS2-Histone 3 and CNS1-H4 acetylation (77, 78). In contrast, phosphorylation and ubiquitination weaken of their suppressive capacity with respective representative mediators Pim-2 kinase and E3 ligase Stub-1 (79, 80).

TREG CELL HETEROGENEITY, PLASTICITY, AND FUNCTIONAL INSTABILITY

It is evident that Foxp3, CD25, co-inhibitory and co-stimulatory molecules, immunosuppressive cytokines, death receptors and fatty-acid oxidation define Treg cell identity and suppressive function. Nevertheless, in recent years it is appreciated that Treg cells present a great phenotypic and functional heterogeneity resulting in distinct Treg cell subsets. These subsets seem not to be terminally differentiated since Treg cells may convert from one subset to another under specific stimuli, in accordance to the notion of plasticity that has been described for T helper (78) cells. Specifically, Treg cells can adopt the transcriptional program and functional characteristics of lineage specific T effector cells under inflammatory conditions (81). Multiple subsets of TH-like Tregs have been reported in cancer and autoimmunity settings expressing transcription factors and characteristic cytokines specific for T effector lineage, such as IFNγ$^+$Tbet$^+$CXCR3$^+$ Th1-like Tregs, IL4$^+$IL5$^+$IL13$^+$GATA3$^+$ Th2-like Tregs, IL17A$^+$RORt$^+$ Th17-like Tregs, and CXCR5+Bcl6+ICOS+PD1+ follicular Tregs (TFR) (82–88). However, the function of the Th-like Treg cells remains controversial (81) since it has been shown that some of these Th-like Treg cells lose their suppressive ability, while others become even more suppressive. For instance, Tbet$^+$ Treg cells colocalized and inhibited Th1 and CD8 T cell activation and elimination of Tbet-expressing Treg cells resulted in severe Th1 autoimmunity. Conversely, in cancer models Tbet$^+$ INFγ$^+$ Tregs lose their suppressive function and promote anti-tumor immune responses (89, 90). Th2-like Tregs were the main Treg subset found in tissues and peripheral blood from patients with colorectal cancer and melanoma compared to healthy individuals displaying high viability, activation and suppressive ability conferring to the tumorigenic environment (87). RORγt$^+$ Treg cells were derived from Foxp3$^+$ thymic Treg cells in an antigen-specific, displayed increased suppressive capacity and efficiently inhibited myelin-specific Th17-cells in a passive experimental autoimmune encephalomyelitis model (91). The main function described for TFR is the suppression of T follicular helper (TFH) cells that support antibody affinity maturation in germinal center reactions and humoral memory formation. Patients with autoimmune rheumatic diseases presented altered numbers of TFR with reduced suppressive function concomitant with a hyperactive phenotype of TFH cells (92). In cancer TFR cells have been found to infiltrate tumors exhibiting superior suppressive capacity and *in vivo* persistence compared to regulatory T cells and their depletion improves tumor control in mice (93). Thus, if the adoption of a Th-like phenotype by Treg cells is a matter of instability or a matter of plasticity remains to be defined. Th-like Treg cells have been characterized as plastic Treg cells in the field of autoimmunity while in cancer they are referred to as fragile Tregs. The common nominator of plastic and fragile Tregs is the production of inflammatory cytokines. In this review we propose the classification of Treg cells into four subpopulations: 1. Treg cells expressing Foxp3, suppressive cytokines and exerting suppressive function, 2. Treg cells expressing Foxp3, producing inflammatory cytokines and retaining their suppressive function, 3. Treg cells expressing Foxp3, producing inflammatory cytokines without exhibiting suppressive function, called from now on fragile Treg cells and 4. the ex-Treg cells that lose Foxp3 expression, produce inflammatory cytokines and do not possess a suppressive function. Both fragile and ex-Treg cells are important players in the pathophysiology of autoimmunity and cancer and will be further reviewed herein (**Figure 1** and **Table 1**).

TREG CELL FUNCTIONAL INSTABILITY IN CANCER

Treg cells highly infiltrate tumors, mediating the formation of an immunosuppressive milieu and thus promoting tumor immune evasion (125). The first evidence regarding their function in inhibiting the anti-tumor immunity emerged 20 years ago, when two independent groups demonstrated that elimination of CD25$^+$CD4$^+$ T cells in mice is associated with enhanced anti-tumor immune responsiveness and tumor regression (126, 127). Several reports demonstrate that an enhanced Treg cell presence in tumor site and peripheral blood of cancer patients was associated with reduced survival and increased metastatic potential in diverse tumor settings (128). On the same line, Treg cell frequencies among tumor infiltrating lymphocytes (TILs) and peripheral blood have been reported to be

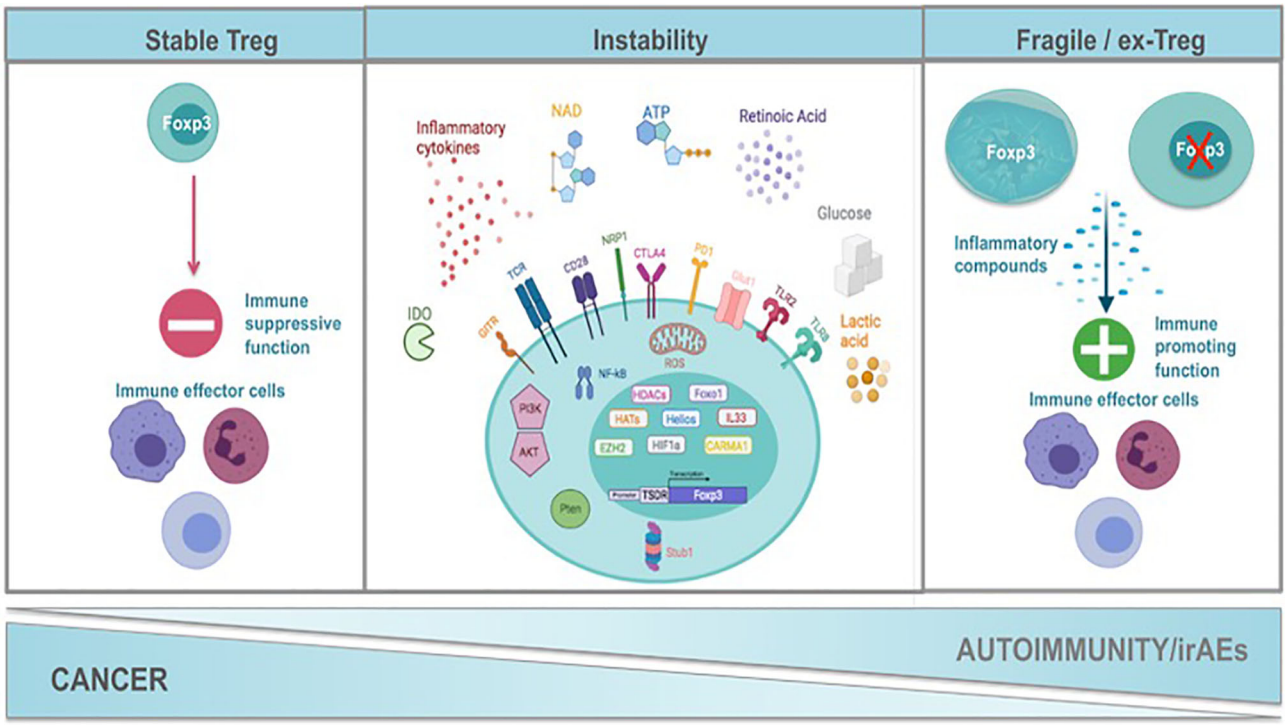


FIGURE 1 | Mechanisms of Treg functional instability constrain cancer while promote autoimmunity and the development of immune related adverse events after immunotherapy. Treg cells exert a strategic role in the maintenance of immune homeostasis. Foxp3 is the hallmark transcription factor of Treg cells orchestrating their function. A complex network of transcription factors such as HELIOS, HIF1a and Foxo1, transcription regulators such as IL33, histone deacetylases, histone methylotransferases, signalosome proteins (CARMA) maintain the transcriptional identity of Tregs and contribute significantly Treg cell stability. Exposure of Tregs to inflammatory stimuli such as inflammatory cytokines (IL1b, IL6, IFNγ, IFNβ, IL12), NAD, ATP and Retinoic Acid results in the alteration of their transcriptional program, in the expression of transcription factors and cytokines that are characteristic of T helper cells and at the end in the attenuation of Treg suppressive function. This may be accompanied by the loss of Foxp3 expression and the generation of ex-Tregs or the generation of fragile Tregs that retain Foxp3 expression. The strength of TCR signaling together with costimulatory and coinhibitory molecules (CD28, GITR, NRP1, CTLA4, PD1) influence downstream pathways such as PI3K/AKT, PTEN, NFkB and the production of ROS by the mitochondria that are critical nominators of the maintenance of Treg transcriptional program and Treg suppressive identity. Oxidative phosphorylation and fatty acid oxidation are metabolic hallmarks of Tregs suppressive function. Any perturbations in the metabolic program of Tregs result in their transformation into non suppressive fragile or ex-Tregs. In autoimmunity the presence of fragile/ex-Tregs burdens the inflammatory response while in cancer stability of Tregs supports the immunosuppressed tumor microenvironment. Immune-checkpoint immunotherapy (ICI) applied in cancer seems to be the destabilization of Tregs and their conversion to fragile or ex-Tregs that may cause the immune-related adverse events (irAEs). Created with BioRender.com.

significantly elevated in nearly all malignancies both in humans and mice, including melanoma, colorectal carcinoma, renal cell carcinoma, pancreatic ductal adenocarcinoma, non-small-cell lung, ovarian epithelial cancer (22, 129–131), gastrointestinal cancer (132), esophageal cancer (133) and breast cancer (134). The accumulation of Treg cells into the tumor niche may involve both the homing of tTreg cells (135), as well as the generation of pTregs (135, 136). The preferential recognition of tumor-specific antigens by the high-affinity TCRs results to clonal expansion, activation and proliferation of Treg cells inside the TME (137).

Many intrinsic and extrinsic factors have been described to induce Treg cell functional instability in the TME. Treg cell lineage specific molecules, TCR/CD28 signaling, metabolism and inflammatory cytokines are factors that have been implicated in the induction of both fragile and ex-Treg cells in cancer resulting in the abolition of the highly immunosuppressive TME and successful control of tumor growth by the immune system. Surprisingly, the same mechanisms that are responsible for the induction of Treg fragility in TME are also those described for the development of ex-Tregs cells (**Figure 1**). The exact mechanisms fine-tuning the decision between the fragile phenotype or the ex-Treg phenotype in the TME are ill defined.

Treg Cell Lineage-Specific Molecules

One of the aforementioned leading mechanisms safeguarding Foxp3 stability is *FOXP3*-TSDR demethylation, which showed significantly higher rates in tumor sites versus normal sites in patients with colorectal cancer. Increased *FOXP3*-TSDR demethylation in combination with a significant upregulation of STAT5, which is an important transcription factor for regulating *FOXP3* expression, resulted in significantly more *FOXP3* mRNA expression and higher protein synthesis in tumor tissues, serving in the pathogenesis of colorectal cancer. *FOXP3*-TSDR demethylation in tumor-infiltrating $CD4^+$ T cells of colorectal cancer patients was mediated by the increase of TET-2 that catalyzed 5-methylcytosine (5mC) conversion to 5-

TABLE 1 | Molecules and mechanisms involved in Treg cell stability in cancer and autoimmunity.

Molecules/Procedures responsible for Treg stability	Mechanism	Disease	Type of instable Treg	Reference
CANCER				
Treg cell lineage-specific molecules				
FOXP3-TSDR methylation	TET-2 MEDIATED 5mC conversion to 5hmC	colorectal cancer	Ex-Tregs	(94)
histone H3K27 methyltransferase of PRC2	EZH2	Colon adenocarcinoma, melanoma, prostate cancer	Ex-Tregs	(95)
bromodomain-containing proteins	interactions of histone acetyl transferases and (HDACs) with transcription factors and proteins involved in gene expression,	Lung adenocarcinoma	Ex-Tregs	(96)
Helios	zinc-finger transcription factors	Melanoma	Ex-Tregs	(97)
Inflammatory cytokines				
IL1b, IL6	Transcription regulator Id2	melanoma	Ex-Tregs	(98)
IFNg		Melanoma	Fragile Th1-like Tregs	(89)
TCR/CD28 signaling pathway				
NF-KB		Melanoma	Ex-Tregs	(96)
IL33	NF-KB-TBET	Melanoma	Fragile Th1-like Tregs	(90)
ROS	BACH2 SUMOylation	Colon carcinoma, melanoma	Ex-Tregs	(99)
Mir-126	PI3K/Akt/mTOR	Breast cancer	Ex-tergs	(100)
Nrp-1	Pten/PI3K/Akt	melanoma	Fragile Th1-like Tregs	(89)
PD-1	Pten/PI3K/Akt	Lung tumor	Fragile Th17-like Tregs	(101)
CARMA-1	AP-1, mTOR, NF-κB	Melanoma, colon carcinoma	Fragile Th1-like Tregs	(102)
Metabolism				
Lactic acid	MCT-1	Melanoma	Tregs with reduced expression of Nrp-1 and elevated levels of PD1	(103)
Autophagy	Atg5, Atg7	Colon adenocarcinoma	Ex-Tregs	(104)
Glycolysis	Traf3ip3	Colon adenocarcinoma	Ex-Tregs	(105)
TLR8	mTOR/glucose metabolism	Melanoma	Tregs with reduced suppressive function	(106)
IDO	GCN2- kinase dependent production of IL-6 by plasmacytoid DC	Melanoma	Fragile Th17-like Tregs	(107)
AUTOIMMUNITY				
Treg cell lineage-specific molecules				
CNS2		Multiple autoimmune diseases	Ex-Tregs	(68, 108)
Stub1/USP7/TIP60/Sirtuin1/ HDAC7	Proteasomal degradation of Foxp3	Multiple autoimmune diseases	Ex-Tregs	(109–112)
Inflammatory cytokines				
IFNβ / IL12 / IFNγ		Multiple sclerosis	Fragile Th1-like Tregs	(113–115)
TCR/CD28 signaling pathway				
PI3K/AKT/Foxo1/3		Multiple sclerosis Multiple autoimmune diseases	Fragile Th1-like Tregs	(116, 117)
PTEN/PI3K/AKT	Glycolysis, Foxo, TSDR methylation	Multiple autoimmune diseases, Multiple sclerosis	Th1-like fragile Tregs	(67, 116–118)
ROS	DNA damage response	Experimental autoimmune encephalomyelitis	Dysfunctional Tregs	(119)
Metabolism				
Extracellular ATP/NAD+	CD39, CD73		ExTregs	(120, 121)
Glut1		Intenstinal inflammation, lupus	ExTregs	(122, 123)
Ubiquitin ligase E3VHL	Glycolysis/ HIF-1a	Multiple autoimmune diseases, Colitis	Th1-like fragile Tregs	(124)

hydroxymethylcytosine (5hmC) (94). Histone modifications are important regulators of chromatin condensation and FOXP3 stability. Pharmacologically or genetically disruption of enhancer of zeste 2 polycomb repressive complex 2 subunit (EZH2) activity, which is a histone H3K27 methyltransferase of the polycomb repressor complex 2 (PRC2) in Treg cells, resulted in the loss of FOXP3 expression and conversion to ex-Tregs cells producing high amounts of pro-inflammatory cytokines, such as

TNF-α, IFN-γ, and IL-2 in the tumor tissues but not in lymphoid organs. The lower Treg cell numbers as well as the acquisition of pro-inflammatory functions of tumor-infiltrating FOXP3$^+$ Treg cells drove to the remodeling of the TME by enhancing the recruitment and function of CD8$^+$ and CD4$^+$ effector T cells and protected mice from colon adenocarcinoma (MC-38), melanoma (B16-F10) and prostate cancer (TRAMP-C2) (95). In accordance with Wang D. et al. (95) results on the role of histone modifications in Treg cells stabilization in the TME Xiong Y. et al. showed that prevention of the recognition of histone modifications by the transcriptional machinery ensued ex-Tregs formation, hindering tumor growth in a genetically engineered mouse model of aggressive lung adenocarcinoma (*Kras*$^{+/LSL\text{-}G12D}$*Trp53*$^{L/L}$ – KP mice). Specifically, treatment of KP mice with JQ1, a well-characterized inhibitor of the bromodomain-containing proteins that modulates the interactions of histone acetyl transferases (96) and histone deacetylases (HDACs) with transcription factors and proteins involved in gene expression, led to a significant downregulation of Foxp3, CTLA-4, and PD-1 only in lung tumor–infiltrated Treg cells accompanied by decreased suppressive function (138). Nevertheless, JQ1 monotherapy led to minimal or moderate delay in tumor growth, but combination treatment with eitherHDACs inhibitor Ricolinostat (138) or anti-PD-1 immunotherapy (139) significantly delayed tumor growth and improved survival of KP mice. Since JQ1 could also induce functional changes to tumor cells the direct and specific role of bromodomains in Treg cell stability in the TME is still debatable.

Helios, which is a member of the Ikaros family of zinc-finger transcription factors and considered as marker of tTreg cells has been shown to play an essential role in the maintenance of Treg cell program. Accordingly, selective depletion of Helios in Treg cells led to enhanced anti-tumor immunity in the B16F10 melanoma model through induction of an unstable Treg cell phenotype in the TME. Helios-deficient tumor-infiltrating Treg cells produced significant amounts of proinflammatory cytokines (TNF-a, IFN-γ), displayed a nonanergic phenotype, reduced immunosuppressive activity and profoundly restrained Foxp3 and CD25 expression (97).

Inflammatory Cytokines

Both pro- and anti- tumorigenic effects have been reported for inflammatory cytokines. These contradictory results may be attributed to the divergent role of cytokines on different cells forming the TME as well as to different effects of cytokines depending on tumor stage. Specifically, for Treg cells it has been shown that exposure to inflammatory cytokines such as IL-1β and IL-6 substantially reduced Treg cell stability and enhanced the conversion of Treg cells to ex-Treg Th17 cells *via* the upregulation of the transcription regulator Id2 (98, 140). Treg cell-specific ectopic expression of Id2 (TetRId2EmGFPFoxp3YFP−Cre) resulted in reduced Foxp3$^+$ Treg cell infiltration within tumor tissue as well as in tumor-draining lymph nodes, increased the expression of IL-17A within the CD4$^+$Foxp3$^-$ tumor TILs and arrested tumor growth in B16F10 melanoma-bearing mice (98). These results implied that the presence of inflammatory cytokines in the immunosuppressed TME further potentiates Treg cells stability and function. Interestingly, Overacre-Delgoffe et al. demonstrated that IFN-γ produced by fragile Treg cells could destabilize the suppressive Treg cells infiltrating tumors, a process named by the authors as "infectious fragility". IFN-γ substantially limited the suppressive capacity of both mouse and human tumor infiltrating Treg cells and not that of peripheral Treg cells (89).

TCR/CD28 Signaling Pathway

TCR and CD28 stimulation facilitates the activation of Treg cell and is indispensable for the preservation of the activated Treg cell transcriptional signature. Nevertheless, the fine-tuning of the strength of TCR stimulation seems to be pivotal for the maintenance of Treg cells. Treg cells possess a plethora of mechanism to attenuate TCR/CD28 signaling including diminished calcium flux, retained activation of Akt, Foxp3-mediated suppression of *Zap70* transcription and expression of inhibitory receptors such as CTLA-4 and CD5 (141). In the TME TCR/CD28 signaling seems also to denominate Treg stability and promote tumor growth.

NF-κB activation is a key downstream event of TCR/CD28 signaling. Activation of NF-κB occurs through the canonical pathway leading to the activation of NF-κB heterodimers consisting of p50 and p65 or p50 and c-Rel and through the non-canonical pathway leading to nuclear translocation of p52-RelB heterodimers. Genetic ablation or chemical inhibition of c-Rel with an FDA-approved drug, Pentoxfylline (PTXF) but not p65 in melanoma-bearing mice modified the transcriptional landscape of activated Treg cells. Specifically, it caused significantly decreased expression of Treg cell markers such as Foxp3, CD25 and Helios and genes required for optimal Treg cell function and immunosuppression in the TME, such as *Tgfb1* or *Gzmb*. Thus, c-Rel inhibition reduced Treg cell activity in the TME resulting in reinforcement of anti-tumor immunity, attenuation of tumor growth and potentiation of anti-PD-1 therapy without causing autoimmunity (96). Nevertheless, we have recently demonstrated that NF-κB pathway is also responsible for the induction of fragile Treg cells in the TME. In detail, specific genetic depletion of IL-33 in Treg cells, which binds to NF-κB and restricts its transcriptional activity, attenuated Treg suppressive properties *in vivo* and facilitated tumor regression in the B16F10 melanoma model. Absence of IL-33, epigenetically reprogrammed Treg cells to express IFN-γ, consistent with a fragile phenotype, dependent on NF-κB–T-bet axis, while maintaining Foxp3 expression. Importantly, genetic ablation of *Il33* potentiated the therapeutic efficacy of immunotherapy (90).

Several studies have suggested that excessive reactive oxygen species (1, 26) levels are associated with tumor-induced immunosuppression and that ROS can participate in Treg cell-mediated immunosuppression. Likewise, ROS that is induced upon TCR and CD28 activation was found to be increased in tumor-infiltrating Treg cells compared to their splenic counterparts. A recent paper in Nature Communications by Yu X. et al. (99) unraveled the molecular mechanism underlying the cross-talk between ROS and Treg cell-mediated

tumor immunosuppression. TCR/CD28 induced ROS led to the accumulation and stabilization of small ubiquitin-related modifier (SUMO)-specific protease 3 (SENP3) in Treg cells repressing T effector cell-specific transcriptional programs and maintaining Treg cell-specific gene signatures by triggering BACH2 deSUMOylation. In detail, genetic deletion of *Senp3* specifically in Treg cells led to the expression of T effector-related genes such as *Ifng*, *Il4*, *Il13*, *Il17a*, *Il22*, and *Il9* and loss of Treg cell-specific genes, such as *Foxp3* and *Pdcd1*. Senp3-induced Treg destabilization resulted in increased frequencies and effector function of $CD4^+$ and $CD8^+$ T effector cells infiltrating the tumors and reduction of tumor growth in MC38 colon carcinoma model and B16F10 melanoma model. These findings suggested that targeting ROS in Treg cells may be an effective approach to ameliorate tumor immune tolerance (99).

It is well accepted that TCR signaling activates the phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt)/mechanistic target of rapamycin (mTOR) pathway in Treg cells but limited activation of this pathway is crucial for Treg cell suppressive function. Micro-RNAs can regulate these pathways, stabilize Treg cells and thus be potential targets of cancer immunotherapy. For instance, silencing of miR-126 on Treg cells enhanced the expression of its target p85b and subsequently altered the activation of PI3K/Akt pathway leading in reduced expression of Foxp3, CTLA-4, GITR, IL-10 and TGF-b on Treg cells. Mir-126KO Treg cells presented impaired suppressive function and promoted a robust anti-tumor immune response that resulted in a diminished tumor growth in a murine breast cancer model (100). PI3K/Akt/mTOR pathway has been also implicated in the induction of fragile Treg cells in the TME. Specifically, it has been demonstrated that Nrp-1, which is constantly expressed by Treg cells reduced Akt signaling following ligation with semaphorin (Sema)4a and Nrp1-Sema4a interaction promoted Treg cell survival, stability transcriptional program with downregulation of the lineage defining transcription factors Eomes, IRF4 and RORγt. Indeed, specific deletion of Nrp-1 in Treg cells ($Nrp1^{f/f}Foxp3^{Cre}$ mice) or blockade of Nrp-1 with Sema4a mAb, Nrp-1 mAb and Sema4a-Ig significantly decreased tumor growth in the B16F10 melanoma mouse model (29, 89). Nrp1KO Treg cells presented a fragile phenotype characterized by expression of IFN-γ, elevated phospho-Akt, reduced ICOS expression and lack of suppressive activity *in vitro* although retaining Foxp3 expression (89). Mechanistically, Nrp-1 recruited the Phosphatase and tensin homolog (PTEN) to the immunologic synapse, which inhibited PI3K and thus limited phosphorylation of Akt (29). PD-1 which is expressed by tumor activated Treg cells is also an upstream regulator of PTEN restricting Akt activation. The *in vitro* blockade of PD-1 pathway in Treg cells rapidly increased Akt phosphorylation, FoxO3a was lost, and suppression activity was abrogated. Thus, it seems that PTEN played an important role in Treg cell function and stability. Indeed, aggressive melanoma and lung tumors implanted in PTEN-Treg-KO hosts grew much slower accompanied by a robust anti-tumor immunity. The tumor-infiltrating PTENKO Treg cells lost the expression of PD-1 but not Foxp3, expressed proinflammatory cytokines such as IL-2 and IL-17 (101).

Finally, the dominant role of TCR/CD28 and downstream molecules regulation in Treg cell functional stability was further confirmed by Di Pilato et al. who studied how the function of TME Treg cells is altered by CARMA1, which is a scaffold protein of the caspase recruitment domain-containing membrane-associated guanylate kinase protein-1 (CARMA1)–B cell lymphoma (BCL)10–Mucosa-associated lymphoid tissue lymphoma translocation protein 1 (MALT1) (CBM) signalosome complex implicated in activation of AP-1, mTOR, NF-κB and mRNA stabilization in response to TCR. $Carma1^{-/-}$ Treg cells retained expression of Foxp3 but secreted IFN-γ and at lower frequencies IL-4, IL-17 and TNF. Production of IFN-γ by Treg cells induced the activation of the intra-tumoral myeloid cells and increased the antigen presenting capacity of tumor cells, resulting in restrained tumor growth. Importantly, blockade of PD-1 in absence of CARMA1 caused rejection of tumors that otherwise do not respond to anti-PD-1 monotherapy (102).

Metabolism

Recent studies have revealed that metabolic programs play pivotal roles in controlling Treg cells stability. Treg cells require predominantly fatty-acid oxidation in contrast to effector T cells that are glycolytic. Interestingly, it has recently been demonstrated that inhibition of lipid synthesis in intra-tumoral Tregs of mouse models diminishes tumor progression, enhancing anti-tumor immune responses (142). Thus, in the harsh TME that it is poor of glucose and high in lactic acid, Treg cells possess a survival and functional advantage promoting immunosuppression. Intratumoral Treg cells are adapted to the lactic-enriched TME by the upregulation of CD36 *via* a peroxisome proliferator-activated receptor-β (PPAR-β) signaling (36). Lactic acid was an additional energy source for Treg cells in the TME, up-taken through transporter monocarboxylate transporter (MCT) 4 and converted to pyruvate and NADH (23). In accordance, glycolysis by tumor cells correlated with the suppressive function of intratumoral Treg cells. Sequencing of tumor-draining lymph node and tumor Treg cells that present high glucose avidity from B16F10 melanoma-bearing mice revealed reduced expression of Treg cell signature genes, while retaining Foxp3 expression. Subsequently, tumor-infiltrating Treg cells that are deficient for MCT1, which catalyzes the intake of lactic acid, upregulated glucose consumption. The MCT1KO Treg cells that present a reduced suppressive function *ex vivo*, decreased expression of Nrp-1 and elevated PD-1 levels lost their suppressive function and allowed the control of B16F10 tumor growth. Treg-specific deletion of the lactate transporter resulted in decreased tumor growth and response to ICI (103).

Autophagy and lysosomal function regulate Treg cell metabolic fitness in the TME. Specifically, Treg cell-specific deletion of the autophagy gene *Atg7* or *Atg5* increased glycolytic metabolism, broke Treg cell stability and facilitated tumor clearance (104). On the other hand, Treg cell–specific

deletion of lysosomal *Traf3ip3* potentiated mTORC1 signaling, mediated hyper-glycolytic metabolism and impaired Treg cell function. Traf3ip3KO Tregs induced a strong anti-tumor T cell response and a profound reduction in tumor size in the MC38 colon carcinoma model. Interestingly, both *Traf3ip3* and *Atg7/Atg5* deficient Treg cells upregulated the expression of inflammatory cytokines genes, such as *Ifng*, *Il4*, *Il13*, *Il17a*, *Il17f*, and *Il21* and presented impaired transcription of the Treg cell signature gene *Foxp3* (105).

Despite the fact that data on mouse tumor Treg cells strongly suggest the use of lipid oxidation as a primary metabolic pathway the same is not true for human Treg cells. The suppressive function of human tumor-associated Treg cells is predominant dependent on glucose metabolism triggering cell senescence and DNA damage in responder T cells. Disruption of glucose metabolism by toll-like receptor (TLR) 8 signaling in human Treg cells reversed Treg inhibitory functions, enhanced anti-tumor immunity and tumor immunotherapy efficacy in a mouse model of melanoma (106).

The consumption of specific nutrients in the TME is another mechanism for the maintenance of immunosuppression and Treg stability. For instance, IDO (19), an enzyme implicated in tryptophan metabolism, was upregulated in murine plasmacytoid DC in tumor-draining lymph nodes, where it potently activated Treg cells. Pharmacological inhibition of IDO in the B16F10 melanoma model released the GCN2-kinase dependent production of IL-6 by plasmacytoid DC and promoted conversion of Treg cells to the Th17-like phenotype. Th17-like Tregs that expressed IL-17, IL-22, IL-2, TNF and RORγt but in parallel maintained Foxp3 expression markedly enhanced anti-tumor immunity (107).

Exhaustion

Another important barely studied category of Treg functional instability in the TME is exhaustion. Treg cells in the peripheral blood and tumor of glioblastoma multiform patients, upregulated the PD-1 concomitantly with IFN-γ and molecular signatures of exhaustion. PD-1 Treg cells presented reduced suppression capacity *in vitro* and a partial demethylation at the TSDR locus while they preserved the FoxP3 expression. These data are in contrast to the aforementioned data about the role of PD-1/PD-L1 axis in promoting Treg induction through inhibition of the Akt/mTOR pathway. Nevertheless, human Treg cells presented a different biology compared to murine Treg cells and also the expression of PD-1 in human Treg cells may be induced as a compensatory mechanism to stabilize the PI3K/Akt pathway and repress IFN-γ (143).

TREG CELL FUNCTIONAL INSTABILITY IN AUTOIMMUNITY

Autoimmune diseases comprise a heterogeneous group of poorly understood long-term disorders that affect approximately 5-8% of the population (144). While each autoimmune disorder is unique, they are all caused by a breakdown of tolerance against endogenous proteins. This leads to auto-inflammatory events that promote the destruction of organs in a humoral and cellular immune mediated manner. Immune suppression by Foxp3$^+$ Treg cells is essential and indispensable for maintenance of tolerance and prevention of autoimmunity, as illustrated by spontaneous autoimmune disease development when Treg cells are rendered deficient. Consistently recent studies have highlighted that Treg dysfunction is a common denominator in autoimmunity, with reduced Treg cell frequencies and impaired suppressive function identified in a wide range of autoimmune diseases, including multiple sclerosis (5), SLE, type 1 diabetes, thyroiditis, and inflammatory bowel disease (145–150). Thus, it is becoming apparent that Treg cells possess a unique power in supervising autoimmune reactions and the re-establishment of self-tolerance. Treg cells during autoimmunity may receive ques from the inflammatory environment that imprint on their phenotype and function, leading to acquisition of an unstable phenotype due to either loss of *Foxp3* expression or fragility with maintenance of *Foxp3* expression (**Figure 1**). An in-depth characterization of the mechanisms underlying Treg cell dysfunction in autoimmunity would enable new strategies for managing autoimmune diseases. In this section we will focus on recent literature exploring Treg cell stability and plasticity and their implications for the pathogenesis of autoimmune diseases.

Ex-Treg Cells in Autoimmunity

Loss of *Foxp3* expression has been shown to contribute to autoimmunity and inflammation in various *in vivo* settings (151–158). Under autoimmune conditions of diabetes, a substantial percentage of cells had unstable expression of *Foxp3* in inflamed tissues. These 'exFoxp3' T cells, secreted inflammatory cytokines, acquired an activated-memory phenotype and were able to induce rapid onset of diabetes upon adoptive transfer (155). In a different autoimmune setting, experimental autoimmune encephalitis, immune activation and inflammation driven by self-antigens in the central nervous system, promoted Foxp3 instability exclusively in autoreactive Treg cells during the induction phase of the response, a process that was reversed during the resolution phase of inflammation or upon IL-2-anti-IL-2 complex treatment (158). Furthermore, data from human studies, highlight the importance of the imbalance of Th17/Treg cell ratio as a pathological feature in multiple sclerosis (5), positively correlating with disease severity (159, 160). In this notion, impaired *Foxp3* and *Helios* expression along with increased numbers of CD161$^+$Th17 like CD45RA$^-$Foxp3^{lo} Treg cells was an early hallmark of multiple sclerosis (161), whereas epigenetic modification of *Foxp3* through histone deacetylase mediated by TLR-2 stimulation induced IL-17 production in Treg cells isolated from multiple sclerosis patients (162). Similarly, Komatsu N. and colleagues demonstrated the pathogenic conversion of Treg cells that lost their *Foxp3* expression into Th17 cells during autoimmune arthritis. Fate mapping analysis showed that IL-17-expressing exFoxp3 T cells accumulated in inflamed joints, expressed Sox4, chemokine (C-C motif) receptor 6 (CCR6), chemokine (C-C motif) ligand 20 (CCL20), IL-23

receptor (IL-23R) and receptor activator of NF-κB ligand (RANKL, also called TNFSF11), in a process mediated by synovial fibroblast-derived IL-6 (153). Among the four CNSs described for the initiation and maintenance of *Foxp3* transcription, CNS2 containing Runx1-CBFβ binding sites, is the only one preventing autoimmunity. In this context, CNS2-deficient mice succumb to development of autoimmunity due to loss of Foxp3 and instability in Treg compartment (68, 108).

Post translational or epigenetic modifications, affect Foxp3 protein expression and thus regulate Treg cell function and development of autoimmunity. Diverse inflammatory stimuli have been shown to promote Lys48-linked ubiquitination mediated by Stub1 ubiquitinase binding to Foxp3, thus targeting it for proteasomal degradation (109). In contrast, under similar inflammatory conditions, USP7 deubiquitinating enzyme expressed in Treg cells, is downregulated resulting in Foxp3 degradation (110), while its conditional deletion in Treg cells leads to lethal autoimmunity (76). Additionally, disruption of the association of other proteins known to mediate Foxp3 acetylation, such as TIP60, Sirtuin 1 or HDAC7, leads to increased polyubiquitination of Foxp3 and development of autoimmune responses (111, 112).

Fragile Treg Cells in Autoimmunity

Fragility of Treg cells has recently arisen as a hallmark of autoimmune diseases, with Treg cells rendered dysfunctional in their suppressive features whilst expressing pro-inflammatory cytokines, maintaining Foxp3 expression and acquiring Th cell-like phenotypes, with identical transcription factors used by Treg cells to inhibit specific types of immune response (84, 113, 116, 117, 163–165).

Up to date, the best characterized Th-like Treg subset in autoimmunity is the Th1-like Treg cells, with upregulated expression of transcription factor Tbet, chemokines CCR5 and CXCR3, stable Foxp3 expression due to highly demethylated TSDR region and increased production of IFN-γ cytokine. Increased frequency of these Th1-like Tregs has been observed in periphery of mouse models and patients with autoimmune diseases, such as type1 diabetes (113, 166), multiple sclerosis (116, 163), autoimmune hepatitis (165) and Sjogren syndrome (167). Following treatment with IFN-β, numbers of IFN-γ secreting Th1-like Treg cells are downregulated to physiological levels in individuals with multiple sclerosis (114). Moreover, blocking IFN-γ is capable of re-establishing Th1-like Treg cells suppressive function during multiple sclerosis in humans and animal models, whereas neutralization of IL-12 resulted in restraining their generation (113, 115). Mechanistically, using a genome wide gene expression approach Kitz et al. demonstrated that PI3K/AKT/Foxo1/3 pathway was responsible for IFN-γ secretion by human Treg cells. Blockade of this pathway, using multiple sclerosis as their *in vivo model*, inhibited IFN-γ secretion and restored the immune suppressive function of Treg cells (116). In the same path, Ouyang W. and colleagues, demonstrated that mice with depleted Foxo1 expression specifically in Treg cells, developed a fatal auto-inflammatory syndrome without the loss of Foxp3 expression and Treg cells displayed a Th1-like phenotype with loss of *in vivo* suppressive activity (117). Importantly, the same study was able to identify approximately 300 Foxo1-bound target genes, including IFN-γ, that were not directly regulated by Foxp3, implying that separate and autonomous signaling pathways may operate simultaneously driving Treg function in autoimmunity.

The second Th-like Treg subset operating in autoimmune diseases is Th17-like Treg cells. Specifically, identification of increased numbers of IL-17$^+$Foxp3$^+$ Treg cells in the synovium of individuals with active rheumatoid arthritis (5), suggests that plastic Foxp3$^+$ Treg cells contribute to the pathogenesis of rheumatoid arthritis (153). Moreover, IL-17A$^+$Foxp3$^+$CD4$^+$ cells have been observed in skin lesions of patients with severe psoriasis (84) and in experimental models of autoimmunity (168). However, observations concerning Th17-like Treg suppressive function isolated from rheumatoid arthritis patients have been rather contradictory depending on the site of Treg cell isolation. To this end, although high frequencies of IL-17-producing Treg cells were present in the peripheral blood of rheumatoid arthritis patients, these cells were able to suppress T cell proliferation *in vitro*. In contrary, Treg cells isolated from rheumatoid arthritis synovial fluid lost their suppressive function (169).

Metabolic Cues in Treg Cell Functional Stability During Autoimmunity

Deficiencies in metabolites such as retinoic acid or vitamin D are prevalent in multiple autoimmune syndromes and are established as a risk factor for development of diseases such as multiple sclerosis, rheumatoid arthritis, SLE and type 1 diabetes (170, 171). The same metabolites, however, have been shown to increase stability of Treg cells in diverse experimental settings. To this end retinoic acid can prevent loss of Foxp3 expression during human Treg expansion and in inflammation (172), it directly increases the expression of ERK signaling to promote *Foxp3* expression as well as increases histone methylation and acetylation of the promoter and CNS region of *Foxp3* (173). In a similar manner, Vitamin D metabolites such as 1,25-dihydroxyvitamin D3, promote *FOXP3* expression by binding to newly identified vitamin D response elements in the intronic CNS region of human *FOXP3* gene (174, 175). From the above-mentioned studies a solid hypothesis is that the lack of essential metabolites from vitamins that is characterizing autoimmune diseases can be detrimental to Treg stability and immunosuppressive function.

Other metabolites derived from tryptophan catabolism, initiated by enzyme IDO, are known to promote *Foxp3* expression, through inhibition of IL-6 production by DC (107, 176), whereas altered tryptophan distribution has been identified in a variety of autoimmune settings (177). Moreover, Foxp3 stability can be also regulated by metabolites deriving from extracellular purine metabolism. Thus, during cell damage and inflammation ATP and NAD$^+$ molecules are released extracellularly due to enhanced cell lysis and are able to activate P2x7 receptor on Treg surface that in terms limits *Foxp3* expression and induces their conversion to TH17 cells (120).

To counterbalance this effect Treg cells are known to express CD39 and CD73 ectonucleotidases on their surface, responsible for converting excess extracellular ATP to adenosine that is immunosuppressive (40). Nevertheless, during autoimmune diseases CD39 and CD73 expression on Treg cells is downregulated possibly providing a link to adenosine and Treg instability in autoimmunity (121).

Cellular metabolism is also closely linked to Treg cell stability and plasticity. As mentioned above Treg cells rely more on mitochondrial metabolism compared to glycolysis to maintain their energy production and suppressive function and *Foxp3* expression orchestrates Treg cell metabolism by suppressing glycolysis and enhancing OXPHOS through mTORC1 (45, 121). In favour of this concept deletion of hypoxia inducible factor (HIF)-1α known to promote glycolysis, leads to increased Foxp3 stability and Treg cell induction (178). In addition, mice overexpressing *Glut1* have reduced *Foxp3* expression during intestinal inflammation (122), while pharmacologic inhibition of Glut1 ameliorates lupus autoimmune phenotype in mice by targeting T cell activation (123). Furthermore, ubiquitin ligase E3VHL deficient Treg cells become IFN-γsecreting Th1-like cells through a shift in glycolysis and increased binding of HIF-1α to *Ifng* promoter (124). Bridging Treg cellular metabolic function to autoimmune pathogenicity, our group found a Treg dysfunction recapitulating the features of autoimmune Treg cells, with a prominent mitochondrial ROS signature and importantly, scavenging of Treg mitochondrial ROS production was able to ameliorate experimental encephalomyelitis in mice (119).

Intracellular signalling pathways involved in Treg cell metabolism also play a crucial role in maintaining their stability and controlling their plasticity. PTEN deletion in Treg cells increases PI3K/AKT pathway activation driving enhanced glycolysis, reduced FoxO presence in the nucleus and promoter regions of *Foxp3* and increased methylation of its TSDR region (67, 117, 118). In parallel, enhanced AKT activation in Treg cells has been demonstrated during autoimmune diseases (116, 179, 180), whereas blockade of this pathway in Treg cells isolated from multiple sclerosis patients inhibits IFN-γ secretion and restores the immune suppressive function of Treg cells (116).

TREG CELL FUNCTIONAL INSTABILITY IN CANCER IMMUNOTHERAPY AND AUTOIMMUNE RELATED ADVERSE EVENTS

Despite of the promising results of cancer immunotherapy, its clinical efficacy is limited to the minority of patients, whereas it is usually accompanied by the development of immune related adverse event (irAEs), due to the excessive activation of the immune system, with the underlying mechanisms remaining unknown. Accumulating evidence suggests that the prevalence of Treg cells inside the TME is associated with tumor progression, as well as the development of acquired resistance to cancer immunotherapy and irAEs development (130, 181). Considering the above, recent therapeutic attempts have been focused on the manipulation of Treg cell-mediated immunosuppression in order to enhance anti-tumor immune responses and improve the clinical outcome of cancer patients. Several strategies for targeting tumor associated Treg cells may involve either direct or indirect approaches, that have been tested clinically or/and preclinically, such as: a. the CD25 targeting for Treg cell depletion with either blocking antibodies or a recombinant protein composed of IL-2 and the active domain of the diphtheria toxin (127, 182–187), b. the targeting of Treg-specific co-inhibitory molecules (CTLA-4, PD-1, TIGIT, VISTA, TIM-3, LAG-3) (188–191), with blocking antibodies to specifically deplete or diminish the suppressive function of Treg cells in the TME (143, 192–196), c. The usage of agonists against GITR (197–199), OX-40 (200, 201) and ICOS (202) can drive the attenuation of Treg cell immunosuppressive activity (203), d. targeting of PI3K signaling (204) or molecules like CD39 and CD73- critical regulators of adenosine pathway (205)-which are definitive of Treg cells behavior in the TME, e. inhibition of vascular endothelial growth factor (VEGF)-VEGF receptor 2 (VEGF-VEFGFR2) pathway, which is implicated in the accumulation of Treg cells, reduced their infiltration to the TME (206, 207), f. inhibition of TGF-β pathway, a major mediator of Treg presence in the TME, can diminish the induction of Treg cells (208, 209).

The ultimate goal would be to specifically deplete Treg cells infiltrating tumors without affecting tumor-reactive effector T cells, while suppressing autoimmunity. Getting a better insight into the mechanisms that induce functional destabilization of Treg cells may allow their exploitation as therapeutic tools. Induction of Treg functional instability may prove a more redundant approach in cancer immunotherapy compared to the targeting of one specific Treg suppressive mechanism and with less autoimmune side effects compared to depletion of Treg cells.

There are several pieces of evidence showing that ICI, specifically, anti-PD-1 and anti-CTLA-4 that are currently used in clinical practice may induce a destabilized phenotype in tumor Treg cells. Specifically, peripheral Tregs from patients suffering from glioblastoma multiform presented an exhausted phenotype and increased expression of IFN-γ following treatment with anti-PD-1 (143). Moreover, PD-1 blockade increased IFN-γ production in the TME and as a consequence drove intratumoral Treg fragility (89). Anti-CTLA-4 also induced fragility in intratumoral Treg cells. Anti-CTLA-4 treated Treg cells promoted CD28 co-stimulation leading to decreased Treg cells suppression and increased glucose consumption. Inhibition of tumor glycolysis elevated available glucose levels in the TME and promoted the ability of CTLA-4 blockade to induce Treg cell fragility associated with IFN-γ production and development of anti-tumor immunity (210). Among several new immunotherapy targets, GITR activation can promote effector T cell function and inhibit Treg cell function. In line, therapeutic application of the agonist anti-GITR monoclonal antibody DTA-1 in B16F10 melanoma-bearing

mice induced regression of tumors accompanied by decreased accumulation of intra-tumor Treg cells due both to loss of *Foxp3* expression and impaired infiltration (211). Complete *Foxp3* loss in intra-tumoral Treg cells correlated with a dramatic decrease in *Helios* expression and was associated with the upregulation of Tbet, Eomes and INF-γ. Interestingly, tumor preconditioning and the TME were essential for GITR dependent modulation of *Foxp3* expression since Treg cells not exposed to the TME did not lose *Foxp3* expression following treatment with DTA-1. Therefore, agonist GITR antibodies are promising immunotherapeutic tools since they may abolish the immunosuppressive TME without ensuing the autoimmune side effects (97, 212).

As thoroughly described in the previous paragraphs of this review Treg functional instability results on the one hand in tumor eradication but on the other hand in autoimmune manifestations. Whether and when ICI-induced Treg functional instability participates in the development of irAEs remains unexplored. In line with this notion our group has recently identified a Th-like inflammatory signature in Treg cells isolated from peripheral blood of individuals with diverse cancer types developing irAEs following immunotherapy with anti-PD-1. This intense transcriptional reprogramming of Treg cells was characterized by enhanced enrichment in transcripts such as *Ifng, Stat1, Rorc* and *Stat3*, supporting the notion of a breakdown in mechanisms of self-tolerance in individuals with solid tumors developing irAEs upon ICI immunotherapy (213). Moreover, human Treg cells isolated from individuals with irAEs experience a robust metabolic reprogramming, enriched in signatures associated with mitochondrial dysfunction and oxidative stress-induced cell death (119, 213).

CONCLUSIONS, CHALLENGES, AND OPEN QUESTIONS

It is well established that Treg cells play a pivotal role in maintenance of immune homeostasis and also appear to regulate the outcome of diverse pathological situations. At the same time, Treg cells can be characterized by an increased plasticity influenced by several parameters such as the cytokine microenvironment, the strength of antigen recognition, the anatomical site that Treg cells reside etc. Shedding light on the mechanisms that underlie the induction of Treg cell plasticity holds tremendous therapeutic potential in cancer in which Treg suppressive function dominates the tumor immune evasion mechanisms, but also in diseases with aberrancy in Treg cell activity such as autoimmunity and transplantation. A major caveat in performing this task, remains the lack of specific markers to precisely distinguish not only Treg cells from T effectors, but also the different subsets of Treg cells. As we discussed above, Treg cells come in various flavors and adopt a different transcriptional program tailored to the specific microenvironment. Thus, single cell (34) genetic (i.e. RNAseq) and epigenetic (i.e. ATACseq) approaches should provide a comprehensive profiling of Treg cells in each context, which may guide the therapeutic decisions and may reveal unique markers to assist the isolation, functional characterization and targeting of these cells. In addition, particular emphasis should be placed on the metabolic profile of the Treg cells, since over the last decade elegant studies highlight that, metabolic cues determine the functional properties of Treg cells. Therefore, identification of metabolites and pathways that interfere with the Treg cells stability program in a disease setting should be determined.

The fact that Treg cells express major checkpoint inhibitors, which constitute therapeutic targets in both solid tumors and hematologic malignancies with impressive results, proposes that Treg cell manipulation could lead to tumor regression. The goal here should be to induce Treg cell fragility or to interfere with Treg cell suppressive function, preferably in an antigen-specific manner, which will allow the re-start of anti-tumor immunity. To achieve this, we should understand the mechanisms that mediate Treg cell fragility and to identify novel molecules/pathways that could be targeted in order to induce fragile or ex-Treg cells. A major challenge, which still remains is the precise targeting of clonal Treg cells to promote tumor regression without disturbing immune homeostasis. One could hypothesize that ICI give rise to the development of the wide spectrum of irAEs since they also imprint on the peripheral pool of Treg cells impairing their suppressive activity. Although, direct proof is still missing, generated data from our group discussed above, favor the hypothesis and highlight the necessity to unravel the *in vivo* mechanisms of Treg cell-mediated suppression and how immunotherapy interferes with them in specific pathogenic contexts.

Characterization of Treg cell fragile program, may also assist in the development of Treg cell therapies in autoimmunity and transplantation. Various clinical trials and preclinical studies highlight the potential of Treg cell adoptive therapies to treat autoimmune pathologies such as type 1 diabetes, SLE and autoimmune central nervous system disease as well as to induce tolerance during solid organ and bone marrow transplantation. One of the major challenges that have impeded the adoption of Treg cell therapies in the clinic is the lack of knowledge on Treg cell stability in the highly inflammatory environments of the aforementioned pathologic conditions. Considering the advances on the genome editing techniques and the success of engineered chimeric antigen receptor T cell therapies, generation of CAR Treg cell-based therapies has been envisioned, aiming to dampen inflammation and to restore immune tolerance. To this end, the ability to introduce multiple editing events per single cells with the CRISPR technologies, set the stage for generation of Treg cells carrying suicide genes which mediate their fragility, along with genes empowering their function, mediate their trafficking and delivering suppressive mediators. Combined with expression of antigen specific receptors, these engineered Treg cells should hold a tremendous therapeutic potential for inflammatory diseases.

Finally, over the last years the appreciation of the Treg cell residency in non-lymphoid tissues (nltTregs) like skin, adipose tissue, lung and bone marrow, with the ability to control local

inflammatory responses and to express diverse transcriptional programs compared to lymphoid tissue Treg cells, have generated new challenges and questions on the Treg cell biology field. In regards to Treg cell stability, it is of interest to be determined whether and how nltTregs respond to local inflammatory and metabolic cues and if this signals imprint on their stability and on *Foxp3* expression. As an example, Treg cells that reside in adipose tissue have been shown to play an important role in controlling adipose tissue inflammation, while their defects are involved in the pathogenesis of obesity-related metabolic disorders. Towards this, inflammatory cytokines and engagement of major metabolic pathways such mTOR/AKT have been shown to drive Treg cell defects in adipose tissues, however the precise mechanisms leading to Treg cell lose and whether this involves induction of fragility and/or ex-Treg development remain to be determined. Overall, addressing such questions may provide novel strategies for combating chronic inflammation and metabolic disorders but also will aid to the design of rational treatments in cancer.

AUTHOR CONTRIBUTIONS

AH, AB, MP, IP AV and TA performed the literature searches and contributed to draft versions of the manuscript. AH and PV wrote and revised the final version of the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the Hellenic Foundation for Research and Innovation (H.F.R.I) and Stavros Niarchos Foundation (S.N.F), grant #1429 to PV. TA is supported by the European Research Council (ERC) under the European Union's Horizon 2020 research and Innovation program (grant agreement no. 947975) and by the Hellenic Foundation for Research and Innovation (H.F.R.I.) under the "2nd Call for H.F.R.I. Research Projects to support Post-Doctoral Researchers" (Project Number: 166).

REFERENCES

1. Weber SE, Harbertson J, Godebu E, Mros GA, Padrick RC, Carson BD, et al. Adaptive Islet-Specific Regulatory CD4 T Cells Control Autoimmune Diabetes and Mediate the Disappearance of Pathogenic Th1 Cells In Vivo. *J Immunol* (2006) 176:4730–9. doi: 10.4049/jimmunol.176.8.4730
2. Sharma A, Rudra D. Regulatory T Cells as Therapeutic Targets and Mediators. *Int Rev Immunol* (2019) 38:183–203. doi: 10.1080/08830185.2019.1621310
3. Fontenot JD, Gavin MA, Rudensky AY. Foxp3 Programs the Development and Function of CD4+CD25+ Regulatory T Cells. *Nat Immunol* (2003) 4:330–6. doi: 10.1038/ni904
4. 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
5. Bennett CL, Christie J, Ramsdell F, Brunkow ME, Ferguson PJ, Whitesell L, 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
6. 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
7. Khattri R, Cox T, Yasayko SA, Ramsdell F. An Essential Role for Scurfin in CD4 +CD25+ T Regulatory Cells. *Nat Immunol* (2003) 4:337–42. doi: 10.1038/ni909
8. Brunkow ME, Jeffery EW, Hjerrild KA, Paeper B, Clark LB, Yasayko SA, et al. Disruption of a New Forkhead/Winged-Helix Protein, Scurfin, Results in the Fatal Lymphoproliferative Disorder of the Scurfy Mouse. *Nat Genet* (2001) 27:68–73. doi: 10.1038/83784
9. Zheng Y, Josefowicz SZ, Kas A, Chu TT, Gavin MA, Rudensky AY. Genome-Wide Analysis of Foxp3 Target Genes in Developing and Mature Regulatory T Cells. *Nature* (2007) 445:936–40. doi: 10.1038/nature05563
10. Kwon HK, Chen HM, Mathis D, Benoist C. Different Molecular Complexes That Mediate Transcriptional Induction and Repression by Foxp3. *Nat Immunol* (2017) 18:1238–48. doi: 10.1038/ni.3835
11. Arvey A, van der Veeken J, Samstein RM, Feng Y, Stamatoyannopoulos JA, Rudensky AY. Inflammation-Induced Repression of Chromatin Bound by the Transcription Factor Foxp3 in Regulatory T Cells. *Nat Immunol* (2014) 15:580–7. doi: 10.1038/ni.2868
12. Gavin MA, Rasmussen JP, Fontenot JD, Vasta V, Manganiello VC, Beavo JA, et al. Foxp3-Dependent Programme of Regulatory T-Cell Differentiation. *Nature* (2007) 445:771–5. doi: 10.1038/nature05543
13. van der Veeken J, Glasner A, Zhong Y, Hu W, Wang ZM, Bou-Puerto R, et al. The Transcription Factor Foxp3 Shapes Regulatory T Cell Identity by Tuning the Activity of Trans-Acting Intermediaries. *Immunity* (2020) 53 (5):971–84.e5. doi: 10.1016/j.immuni.2020.10.010
14. 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.
15. Almeida AR, Legrand N, Papiernik M, Freitas AA. Homeostasis of Peripheral CD4+ T Cells: IL-2R Alpha and IL-2 Shape a Population of Regulatory Cells That Controls CD4+ T Cell Numbers. *J Immunol* (2002) 169:4850–60. doi: 10.4049/jimmunol.169.9.4850
16. Lu L, Barbi J, Pan F. The Regulation of Immune Tolerance by FOXP3. *Nat Rev Immunol* (2017) 17:703–17. doi: 10.1038/nri.2017.75
17. Alissafi T, Hatzioannou A, Legaki AI, Varveri A, Verginis P. Balancing Cancer Immunotherapy and Immune-Related Adverse Events: The Emerging Role of Regulatory T Cells. *J Autoimmun* (2019) 104:102310. doi: 10.1016/j.jaut.2019.102310
18. Bu M, Shen Y, Seeger WL, An S, Qi R, Sanderson JA, et al. Ovarian Carcinoma-Infiltrating Regulatory T Cells Were More Potent Suppressors of CD8(+) T Cell Inflammation Than Their Peripheral Counterparts, a Function Dependent on TIM3 Expression. *Tumour Biol* (2016) 37:3949–56. doi: 10.1007/s13277-015-4237-x
19. Mittal SK, Cho KJ, Ishido S, Roche PA. Interleukin 10 (IL-10)-Mediated Immunosuppression: March-I Induction Regulates Antigen Presentation by Macrophages But Not Dendritic Cells. *J Biol Chem* (2015) 290:27158–67. doi: 10.1074/jbc.M115.682708
20. Asseman C, Mauze S, Leach MW, Coffman RL, Powrie F. An Essential Role for Interleukin 10 in the Function of Regulatory T Cells That Inhibit Intestinal Inflammation. *J Exp Med* (1999) 190:995–1004. doi: 10.1084/jem.190.7.995
21. Mann MK, Maresz K, Shriver LP, Tan Y, Dittel BN. B Cell Regulation of CD4+CD25+ T Regulatory Cells and IL-10 via B7 Is Essential for Recovery From Experimental Autoimmune Encephalomyelitis. *J Immunol* (2007) 178:3447–56. doi: 10.4049/jimmunol.178.6.3447
22. Sakaguchi S, Miyara M, Costantino CM, Hafler DA. FOXP3+ Regulatory T Cells in the Human Immune System. *Nat Rev Immunol* (2010) 10:490–500. doi: 10.1038/nri2785
23. Wang H, Franco F, Ho PC. Metabolic Regulation of Tregs in Cancer: Opportunities for Immunotherapy. *Trends Cancer* (2017) 3:583–92. doi: 10.1016/j.trecan.2017.06.005

24. Fahlen L, Read S, Gorelik L, Hurst SD, Coffman RL, Flavell RA, et al. T Cells That Cannot Respond to TGF-Beta Escape Control by CD4(+)CD25(+) Regulatory T Cells. *J Exp Med* (2005) 201:737–46. doi: 10.1084/jem.20040685
25. Paluskievicz CM, Cao X, Abdi R, Zheng P, Liu Y, Bromberg JS. T Regulatory Cells and Priming the Suppressive Tumor Microenvironment. *Front Immunol* (2019) 10:2453–2507. doi: 10.3389/fimmu.2019.02453
26. Grossman WJ, Verbsky JW, Barchet W, Colonna M, Atkinson JP, Ley TJ. Human T Regulatory Cells can Use the Perforin Pathway to Cause Autologous Target Cell Death. *Immunity* (2004) 21:589–601. doi: 10.1016/j.immuni.2004.09.002
27. Ren X, Ye F, Jiang Z, Chu Y, Xiong S, Wang Y. Involvement of Cellular Death in TRAIL/DR5-Dependent Suppression Induced by CD4(+)CD25(+) Regulatory T Cells. *Cell Death Differ* (2007) 14:2076–84. doi: 10.1038/sj.cdd.4402220
28. Bodmer JL, Holler N, Reynard S, Vinciguerra P, Schneider P, Juo P, et al. TRAIL Receptor-2 Signals Apoptosis Through FADD and Caspase-8. *Nat Cell Biol* (2000) 2:241–3. doi: 10.1038/35008667
29. Delgoffe GM, Woo SR, Turnis ME, Gravano DM, Guy C, Overacre AE, et al. Stability and Function of Regulatory T Cells Is Maintained by a Neuropilin-1-Semaphorin-4a Axis. *Nature* (2013) 501:252–6. doi: 10.1038/nature12428
30. Cao X, Cai SF, Fehniger TA, Song J, Collins LI, Piwnica-Worms DR, et al. Granzyme B and Perforin are Important for Regulatory T Cell-Mediated Suppression of Tumor Clearance. *Immunity* (2007) 27:635–46. doi: 10.1016/j.immuni.2007.08.014
31. 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
32. Galgani M, De Rosa V, La Cava A, Matarese G. Role of Metabolism in the Immunobiology of Regulatory T Cells. *J Immunol* (2016) 197:2567–75. doi: 10.4049/jimmunol.1600242
33. Angelin A, Gil-de-Gomez L, Dahiya S, Jiao J, Guo L, Levine MH, et al. Foxp3 Reprograms T Cell Metabolism to Function in Low-Glucose, High-Lactate Environments. *Cell Metab* (2017) 5(6):1282–93.e7. doi: 10.1016/j.cmet.2016.12.018
34. McKarns SC, Schwartz RH, Kaminski NE. Smad3 is Essential for TGF-Beta 1 to Suppress IL-2 Production and TCR-Induced Proliferation, But Not IL-2-Induced Proliferation. *J Immunol* (2004) 172:4275–84. doi: 10.4049/jimmunol.172.7.4275
35. Pandiyan P, Zheng L, Ishihara S, Reed J, Lenardo MJ. CD4+CD25+Foxp3+ Regulatory T Cells Induce Cytokine Deprivation-Mediated Apoptosis of Effector CD4+ T Cells. *Nat Immunol* (2007) 8:1353–62. doi: 10.1038/ni1536
36. Wang H, Franco F, Tsui YC, Xie X, Trefny MP, Zappasodi R, et al. CD36-Mediated Metabolic Adaptation Supports Regulatory T Cell Survival and Function in Tumors. *Nat Immunol* (2020) 21:298–308. doi: 10.1038/s41590-019-0589-5
37. Deaglio S, Dwyer KM, Gao W, Friedman D, Usheva A, Erat A, et al. Adenosine Generation Catalyzed by CD39 and CD73 Expressed on Regulatory T Cells Mediates Immune Suppression. *J Exp Med* (2007) 204:1257–65. doi: 10.1084/jem.20062512
38. Wilson JM, Ross WG, Agbai ON, Frazier R, Figler RA, Rieger J, et al. The A2B Adenosine Receptor Impairs the Maturation and Immunogenicity of Dendritic Cells. *J Immunol* (2009) 182:4616–23. doi: 10.4049/jimmunol.0801279
39. Naganuma M, Wiznerowicz EB, Lappas CM, Linden J, Worthington MT, Ernst PB. Cutting Edge: Critical Role for A2A Adenosine Receptors in the T Cell-Mediated Regulation of Colitis. *J Immunol* (2006) 177:2765–9. doi: 10.4049/jimmunol.177.5.2765
40. Borsellino G, Kleinewietfeld M, Di Mitri D, Sternjak A, Diamantini A, Giometto R, et al. Expression of Ectonucleotidase CD39 by Foxp3+ Treg Cells: Hydrolysis of Extracellular ATP and Immune Suppression. *Blood* (2007) 110:1225–32. doi: 10.1182/blood-2006-12-064527
41. Walker LS, 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
42. Alissafi T, Hatzioannou A, Ioannou M, Sparwasser T, Grun JR, Grutzkau A, et al. De Novo-Induced Self-Antigen-Specific Foxp3+ Regulatory T Cells Impair the Accumulation of Inflammatory Dendritic Cells in Draining Lymph Nodes. *J Immunol* (2015) 194:5812–24. doi: 10.4049/jimmunol.1500111
43. Ovcinnikovs V, Ross EM, Petersone L, Edner NM, Heuts F, Ntavli E, et al. CTLA-4-Mediated Transendocytosis of Costimulatory Molecules Primarily Targets Migratory Dendritic Cells. *Sci Immunol* (2019) 4:eaaw0902. doi: 10.1126/sciimmunol.aaw0902
44. Onishi Y, Fehervari Z, Yamaguchi T, Sakaguchi S. Foxp3+ Natural Regulatory T Cells Preferentially Form Aggregates on Dendritic Cells In Vitro and Actively Inhibit Their Maturation. *Proc Natl Acad Sci USA* (2008) 105:10113–8. doi: 10.1073/pnas.0711106105
45. Zeng H, Yang K, Cloer C, Neale G, Vogel P, Chi H. Mtorc1 Couples Immune Signals and Metabolic Programming to Establish T(reg)-Cell Function. *Nature* (2013) 499:485–90. doi: 10.1038/nature12297
46. Alissafi T, Banos A, Boon L, Sparwasser T, Ghigo A, Wing K, et al. Tregs Restrain Dendritic Cell Autophagy to Ameliorate Autoimmunity. *J Clin Invest* (2017) 127:2789–804. doi: 10.1172/JCI92079
47. Sarris M, Andersen KG, Randow F, Mayr L, Betz AG. Neuropilin-1 Expression on Regulatory T Cells Enhances Their Interactions With Dendritic Cells During Antigen Recognition. *Immunity* (2008) 28:402–13. doi: 10.1016/j.immuni.2008.01.012
48. Liang B, Workman C, Lee J, Chew C, Dale BM, Colonna L, et al. Regulatory T Cells Inhibit Dendritic Cells by Lymphocyte Activation Gene-3 Engagement of MHC Class II. *J Immunol* (2008) 180:5916–26. doi: 10.4049/jimmunol.180.9.5916
49. Yang K, Blanco DB, Neale G, Vogel P, Avila J, Clish CB, et al. Homeostatic Control of Metabolic and Functional Fitness of Treg Cells by LKB1 Signalling. *Nature* (2017) 548:602–6. doi: 10.1038/nature23665
50. Huynh A, DuPage M, Priyadharshini B, Sage PT, Quiros J, Borges CM, et al. Control of PI(3) Kinase in Treg Cells Maintains Homeostasis and Lineage Stability. *Nat Immunol* (2015) 16:188–96. doi: 10.1038/ni.3077
51. Gerriets VA, Kishton RJ, Johnson MO, Cohen S, Siska PJ, Nichols AG, et al. Foxp3 and Toll-Like Receptor Signaling Balance Treg Cell Anabolic Metabolism for Suppression. *Nat Immunol* (2016) 17:1459–66. doi: 10.1038/ni.3577
52. Weinberg SE, Singer BD, Steinert EM, Martinez CA, Mehta MM, Martinez-Reyes I, et al. Mitochondrial Complex III Is Essential for Suppressive Function of Regulatory T Cells. *Nature* (2019) 565:495–9. doi: 10.1038/s41586-018-0846-z
53. He N, Fan W, Henriquez B, Yu RT, Atkins AR, Liddle C, et al. Metabolic Control of Regulatory T Cell (Treg) Survival and Function by Lkb1. *Proc Natl Acad Sci USA* (2017) 114:12542–7. doi: 10.1073/pnas.1715363114
54. Newton R, Priyadharshini B, Turka LA. Immunometabolism of Regulatory T Cells. *Nat Immunol* (2016) 17:618–25. doi: 10.1038/ni.3466
55. Pacella I, Procaccini C, Focaccetti C, Miacci S, Timperi E, Faicchia D, et al. Fatty Acid Metabolism Complements Glycolysis in the Selective Regulatory T Cell Expansion During Tumor Growth. *Proc Natl Acad Sci USA* (2018) 115:E6546–E55. doi: 10.1073/pnas.1720113115
56. Zheng Y, Josefowicz S, Chaudhry A, Peng XP, Forbush K, Rudensky AY. Role of Conserved Non-Coding DNA Elements in the Foxp3 Gene in Regulatory T-Cell Fate. *Nature* (2010) 463:808–12. doi: 10.1038/nature08750
57. Kitagawa Y, Ohkura N, Kidani Y, Vandenbon A, Hirota K, Kawakami R, et al. Guidance of Regulatory T Cell Development by Satb1-Dependent Super-Enhancer Establishment. *Nat Immunol* (2017) 18:173–83. doi: 10.1038/ni.3646
58. Baron U, Floess S, Wieczorek G, Baumann K, Grutzkau A, Dong J, et al. DNA Demethylation in the Human FOXP3 Locus Discriminates Regulatory T Cells From Activated FOXP3(+) Conventional T Cells. *Eur J Immunol* (2007) 37:2378–89. doi: 10.1002/eji.200737594
59. Kim HP, Leonard WJ. CREB/ATF-Dependent T Cell Receptor-Induced FoxP3 Gene Expression: A Role for DNA Methylation. *J Exp Med* (2007) 204:1543–51. doi: 10.1084/jem.20070109
60. Mantel PY, Ouaked N, Ruckert B, Karagiannidis C, Welz R, Blaser K, et al. Molecular Mechanisms Underlying FOXP3 Induction in Human T Cells. *J Immunol* (2006) 176:3593–602. doi: 10.4049/jimmunol.176.6.3593
61. Zorn E, Nelson EA, Mohseni M, Porcheray F, Kim H, Litsa D, et al. IL-2 Regulates FOXP3 Expression in Human CD4+CD25+ Regulatory T Cells Through a STAT-Dependent Mechanism and Induces the Expansion of

These Cells In Vivo. *Blood* (2006) 108:1571–9. doi: 10.1182/blood-2006-02-004747
62. Floess S, Freyer J, Siewert C, Baron U, Olek S, Polansky J, et al. Epigenetic Control of the Foxp3 Locus in Regulatory T Cells. *PloS Biol* (2007) 5:e38. doi: 10.1371/journal.pbio.0050038
63. Wieczorek G, Asemissen A, Model F, Turbachova I, Floess S, Liebenberg V, et al. Quantitative DNA Methylation Analysis of FOXP3 as a New Method for Counting Regulatory T Cells in Peripheral Blood and Solid Tissue. *Cancer Res* (2009) 69:599–608. doi: 10.1158/0008-5472.CAN-08-2361
64. Kawakami R, Kitagawa Y, Chen KY, Arai M, Ohara D, Nakamura Y, et al. Distinct Foxp3 Enhancer Elements Coordinate Development, Maintenance, and Function of Regulatory T Cells. *Immunity* (2021) 54(5):947–61.e8. doi: 10.1016/j.immuni.2021.04.005
65. Tone Y, Furuuchi K, Kojima Y, Tykocinski ML, Greene MI, Tone M. SMAD3 and NFAT Cooperate to Induce Foxp3 Expression Through Its Enhancer. *Nat Immunol* (2008) 9:194–202. doi: 10.1038/ni1549
66. Harada Y, Harada Y, Elly C, Ying G, Paik JH, DePinho RA, et al. Transcription Factors Foxo3a and Foxo1 Couple the E3 Ligase Cbl-B to the Induction of Foxp3 Expression in Induced Regulatory T Cells. *J Exp Med* (2010) 207:1381–91. doi: 10.1084/jem.20100004
67. Ouyang W, Beckett O, Ma Q, Paik JH, DePinho RA, Li MO. Foxo Proteins Cooperatively Control the Differentiation of Foxp3+ Regulatory T Cells. *Nat Immunol* (2010) 11:618–27. doi: 10.1038/ni.1884
68. Feng Y, Arvey A, Chinen T, van der Veeken J, Gasteiger G, Rudensky AY. Control of the Inheritance of Regulatory T Cell Identity by a Cis Element in the Foxp3 Locus. *Cell* (2014) 158:749–63. doi: 10.1016/j.cell.2014.07.031
69. Polansky JK, Schreiber L, Thelemann C, Ludwig L, Kruger M, Baumgrass R, et al. Methylation Matters: Binding of Ets-1 to the Demethylated Foxp3 Gene Contributes to the Stabilization of Foxp3 Expression in Regulatory T Cells. *J Mol Med (Berl)* (2010) 88:1029–40. doi: 10.1007/s00109-010-0642-1
70. Sasidharan Nair V, Song MH, Oh KI. Vitamin C Facilitates Demethylation of the Foxp3 Enhancer in a Tet-Dependent Manner. *J Immunol* (2016) 196:2119–31. doi: 10.4049/jimmunol.1502352
71. Gu J, Ni X, Pan X, Lu H, Lu Y, Zhao J, et al. Human CD39(hi) Regulatory T Cells Present Stronger Stability and Function Under Inflammatory Conditions. *Cell Mol Immunol* (2017) 14:521–8. doi: 10.1038/cmi.2016.30
72. Arroyo Hornero R, Betts GJ, Sawitzki B, Vogt K, Harden PN, Wood KJ. CD45RA Distinguishes CD4+CD25+CD127-/Low TSDR Demethylated Regulatory T Cell Subpopulations With Differential Stability and Susceptibility to Tacrolimus-Mediated Inhibition of Suppression. *Transplantation* (2017) 101:302–9. doi: 10.1097/TP.0000000000001278
73. Ohkura N, Kitagawa Y, Sakaguchi S. Development and Maintenance of Regulatory T Cells. *Immunity* (2013) 38:414–23. doi: 10.1016/j.immuni.2013.03.002
74. Ohkura N, Hamaguchi M, Morikawa H, Sugimura K, Tanaka A, Ito Y, et al. T Cell Receptor Stimulation-Induced Epigenetic Changes and Foxp3 Expression are Independent and Complementary Events Required for Treg Cell Development. *Immunity* (2012) 37:785–99. doi: 10.1016/j.immuni.2012.09.010
75. Song X, Li B, Xiao Y, Chen C, Wang Q, Liu Y, et al. Structural and Biological Features of FOXP3 Dimerization Relevant to Regulatory T Cell Function. *Cell Rep* (2012) 1:665–75. doi: 10.1016/j.celrep.2012.04.012
76. Wang L, Kumar S, Dahiya S, Wang F, Wu J, Newick K, et al. Ubiquitin-Specific Protease-7 Inhibition Impairs Tip60-Dependent Foxp3+ T-Regulatory Cell Function and Promotes Antitumor Immunity. *EBioMedicine* (2016) 13:99–112. doi: 10.1016/j.ebiom.2016.10.018
77. Li C, Jiang S, Liu SQ, Lykken E, Zhao LT, Sevilla J, et al. MeCP2 Enforces Foxp3 Expression to Promote Regulatory T Cells' Resilience to Inflammation. *Proc Natl Acad Sci USA* (2014) 111:E2807–16. doi: 10.1073/pnas.1401505111
78. Wu C, Thalhamer T, Franca RF, Xiao S, Wang C, Hotta C, et al. Galectin-9-CD44 Interaction Enhances Stability and Function of Adaptive Regulatory T Cells. *Immunity* (2014) 41:270–82. doi: 10.1016/j.immuni.2014.06.011
79. Morawski PA, Mehra P, Chen C, Bhatti T, Wells AD. Foxp3 Protein Stability Is Regulated by Cyclin-Dependent Kinase 2. *J Biol Chem* (2013) 288:24494–502. doi: 10.1074/jbc.M113.467704
80. Zhang Y, Chen Z, Luo X, Wu B, Li B, Wang B. Cimetidine Down-Regulates Stability of Foxp3 Protein via Stub1 in Treg Cells. *Hum Vaccin Immunother* (2016) 12:2512–8. doi: 10.1080/21645515.2016.1191719
81. 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) 10:595–602. doi: 10.1038/ni.1731
82. 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
83. 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
84. Bovenschen HJ, van de Kerkhof PC, van Erp PE, Woestenenk R, Joosten I, Koenen HJ. Foxp3+ Regulatory T Cells of Psoriasis Patients Easily Differentiate Into IL-17A-Producing Cells and Are Found in Lesional Skin. *J Invest Dermatol* (2011) 131:1853–60. doi: 10.1038/jid.2011.139
85. 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
86. 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
87. Halim L, Romano M, McGregor R, Correa I, Pavlidis P, Grageda N, et al. An Atlas of Human Regulatory T Helper-Like Cells Reveals Features of Th2-Like Tregs That Support a Tumorigenic Environment. *Cell Rep* (2017) 20:757–70. doi: 10.1016/j.celrep.2017.06.079
88. Voo KS, Wang YH, Santori FR, Boggiano C, Wang YH, Arima K, et al. Identification of IL-17-Producing FOXP3+ Regulatory T Cells in Humans. *Proc Natl Acad Sci USA* (2009) 106:4793–8. doi: 10.1073/pnas.0900408106
89. Overacre-Delgoffe AE, Chikina M, Dadey RE, Yano H, Brunazzi EA, Shayan G, et al. Interferon-Gamma Drives Treg Fragility to Promote Anti-Tumor Immunity. *Cell* (2017) 169(6):1130–41.e11. doi: 10.1016/j.cell.2017.05.005
90. Hatzioannou A, Banos A, Sakelaropoulos T, Fedonidis C, Vidali MS, Kohne M, et al. An Intrinsic Role of IL-33 in Treg Cell-Mediated Tumor Immunoevasion. *Nat Immunol* (2020) 21:75–85. doi: 10.1038/s41590-019-0555-2
91. Kim BS, Lu H, Ichiyama K, Chen X, Zhang YB, Mistry NA, et al. Generation of RORgammat(+) Antigen-Specific T Regulatory 17 Cells From Foxp3(+) Precursors in Autoimmunity. *Cell Rep* (2017) 21:195–207. doi: 10.1016/j.celrep.2017.09.021
92. 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
93. Eschweiler S, Clarke J, Ramirez-Suastegui C, Panwar B, Madrigal A, Chee SJ, et al. Intratumoral Follicular Regulatory T Cells Curtail Anti-PD-1 Treatment Efficacy. *Nat Immunol* (2021) 22:1052–63. doi: 10.1038/s41590-021-00958-6
94. Ma H, Gao W, Sun X, Wang W. STAT5 and TET2 Cooperate to Regulate FOXP3-TSDR Demethylation in CD4(+) T Cells of Patients With Colorectal Cancer. *J Immunol Res* (2018) 2018:6985031. doi: 10.1155/2018/6985031
95. Wang D, Quiros J, Mahuron K, Pai CC, Ranzani V, Young A, et al. Targeting EZH2 Reprograms Intratumoral Regulatory T Cells to Enhance Cancer Immunity. *Cell Rep* (2018) 23:3262–74. doi: 10.1016/j.celrep.2018.05.050
96. Grinberg-Bleyer Y, Oh H, Desrichard A, Bhatt DM, Caron R, Chan TA, et al. NF-kappaB C-Rel Is Crucial for the Regulatory T Cell Immune Checkpoint in Cancer. *Cell* (2017) 170(6):1096–108.e13. doi: 10.1016/j.cell.2017.08.004
97. Nakagawa H, Sido JM, Reyes EE, Kiers V, Cantor H, Kim HJ. Instability of Helios-Deficient Tregs is Associated With Conversion to a T-Effector Phenotype and Enhanced Antitumor Immunity. *Proc Natl Acad Sci USA* (2016) 113:6248–53. doi: 10.1073/pnas.1604765113
98. Hwang SM, Sharma G, Verma R, Byun S, Rudra D, Im SH. Inflammation-Induced Id2 Promotes Plasticity in Regulatory T Cells. *Nat Commun* (2018) 9:4736–69. doi: 10.1038/s41467-018-07254-2
99. Yu X, Lao Y, Teng XL, Li S, Zhou Y, Wang F, et al. SENP3 Maintains the Stability and Function of Regulatory T Cells via BACH2 Desumoylation. *Nat Commun* (2018) 9:3157–89. doi: 10.1038/s41467-018-05676-6
100. Qin A, Wen Z, Zhou Y, Li Y, Li Y, Luo J, et al. MicroRNA-126 Regulates the Induction and Function of CD4(+) Foxp3(+) Regulatory T Cells Through

PI3K/AKT Pathway. *J Cell Mol Med* (2013) 17:252–64. doi: 10.1111/jcmm.12003
101. Sharma MD, Shinde R, McGaha TL, Huang L, Holmgaard RB, Wolchok JD, et al. The PTEN Pathway in Tregs Is a Critical Driver of the Suppressive Tumor Microenvironment. *Sci Adv* (2015) 1:e1500845. doi: 10.1126/sciadv.1500845
102. Di Pilato M, Kim EY, Cadilha BL, Prussmann JN, Nasrallah MN, Seruggia D, et al. Targeting the CBM Complex Causes Treg Cells to Prime Tumours for Immune Checkpoint Therapy. *Nature* (2019) 570:112–6. doi: 10.1038/s41586-019-1215-2
103. Watson MJ, Vignali PDA, Mullett SJ, Overacre-Delgoffe AE, Peralta RM, Grebinoski S, et al. Metabolic Support of Tumour-Infiltrating Regulatory T Cells by Lactic Acid. *Nature* (2021) 591:645–51. doi: 10.1038/s41586-020-03045-2
104. Wei J, Long L, Yang K, Guy C, Shrestha S, Chen Z, et al. Autophagy Enforces Functional Integrity of Regulatory T Cells by Coupling Environmental Cues and Metabolic Homeostasis. *Nat Immunol* (2016) 17:277–85. doi: 10.1038/ni.3365
105. Yu X, Teng XL, Wang F, Zheng Y, Qu G, Zhou Y, et al. Metabolic Control of Regulatory T Cell Stability and Function by TRAF3IP3 at the Lysosome. *J Exp Med* (2018) 215:2463–76. doi: 10.1084/jem.20180397
106. Li L, Liu X, Sanders KL, Edwards JL, Ye J, Si F, et al. TLR8-Mediated Metabolic Control of Human Treg Function: A Mechanistic Target for Cancer Immunotherapy. *Cell Metab* (2019) 29(1):103–23.e5. doi: 10.1016/j.cmet.2018.09.020
107. Sharma MD, Hou DY, Liu Y, Koni PA, Metz R, Chandler P, et al. Indoleamine 2,3-Dioxygenase Controls Conversion of Foxp3+ Tregs to TH17-Like Cells in Tumor-Draining Lymph Nodes. *Blood* (2009) 113:6102–11. doi: 10.1182/blood-2008-12-195354
108. Li X, Liang Y, LeBlanc M, Benner C, Zheng Y. Function of a Foxp3 Cis-Element in Protecting Regulatory T Cell Identity. *Cell* (2014) 158:734–48. doi: 10.1016/j.cell.2014.07.030
109. Chen Z, Barbi J, Bu S, Yang HY, Li Z, Gao Y, et al. The Ubiquitin Ligase Stub1 Negatively Modulates Regulatory T Cell Suppressive Activity by Promoting Degradation of the Transcription Factor Foxp3. *Immunity* (2013) 39:272–85. doi: 10.1016/j.immuni.2013.08.006
110. van Loosdregt J, Vercoulen Y, Guichelaar T, Gent YY, Beekman JM, van Beekum O, et al. Regulation of Treg Functionality by Acetylation-Mediated Foxp3 Protein Stabilization. *Blood* (2010) 115:965–74. doi: 10.1182/blood-2009-02-207118
111. Bettini ML, Pan F, Bettini M, Finkelstein D, Rehg JE, Floess S, et al. Loss of Epigenetic Modification Driven by the Foxp3 Transcription Factor Leads to Regulatory T Cell Insufficiency. *Immunity* (2012) 36:717–30. doi: 10.1016/j.immuni.2012.03.020
112. Zhang Y, Wu BX, Metelli A, Thaxton JE, Hong F, Rachidi S, et al. GP96 is a GARP Chaperone and Controls Regulatory T Cell Functions. *J Clin Invest* (2015) 125:859–69. doi: 10.1172/JCI79014
113. McClymont SA, Putnam AL, Lee MR, Esensten JH, Liu W, Hulme MA, et al. Plasticity of Human Regulatory T Cells in Healthy Subjects and Patients With Type 1 Diabetes. *J Immunol* (2011) 186:3918–26. doi: 10.4049/jimmunol.1003099
114. Rakebrandt N, Littringer K, Joller N. Regulatory T Cells: Balancing Protection Versus Pathology. *Swiss Med Wkly* (2016) 146:w14343. doi: 10.4414/smw.2016.14343
115. Piconese S, Timperi E, Barnaba V. 'Hardcore' Ox40 + Immunosuppressive Regulatory T Cells in Hepatic Cirrhosis and Cancer. *Oncoimmunology* (2014) 3:e29257. doi: 10.4161/onci.29257
116. Kitz A, de Marcken M, Gautron AS, Mitrovic M, Hafler DA, Dominguez-Villar M. AKT Isoforms Modulate Th1-Like Treg Generation and Function in Human Autoimmune Disease. *EMBO Rep* (2016) 17:1169–83. doi: 10.15252/embr.201541905
117. 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
118. Newton RH, Shrestha S, Sullivan JM, Yates KB, Compeer EB, Ron-Harel N, et al. Maintenance of CD4 T Cell Fitness Through Regulation of Foxo1. *Nat Immunol* (2018) 19:838–48. doi: 10.1038/s41590-018-0157-4
119. Alissafi T, Kalafati L, Lazari M, Filia A, Kloukina I, Manifava M, et al. Mitochondrial Oxidative Damage Underlies Regulatory T Cell Defects in Autoimmunity. *Cell Metab* (2020) 32(4):591–604.e7. doi: 10.1016/j.cmet.2020.07.001
120. 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
121. Magni G, Ceruti S. Adenosine Signaling in Autoimmune Disorders. *Pharm (Basel)* (2020) 13:260–311. doi: 10.3390/ph13090260
122. Gerriets VA, Kishton RJ, Johnson MO, Cohen S, Siska PJ, Nichols AG, et al. Foxp3 and Toll-Like Receptor Signaling Balance T. *Nat Immunol* (2016) 17:1459–66. doi: 10.1038/ni.3577
123. Li W, Qu G, Choi SC, Cornaby C, Titov A, Kanda N, et al. Targeting T Cell Activation and Lupus Autoimmune Phenotypes by Inhibiting Glucose Transporters. *Front Immunol* (2019) 10:833. doi: 10.3389/fimmu.2019.00833
124. Lee JH, Elly C, Park Y, Liu YC. E3 Ubiquitin Ligase VHL Regulates Hypoxia-Inducible Factor-1α to Maintain Regulatory T Cell Stability and Suppressive Capacity. *Immunity* (2015) 42:1062–74. doi: 10.1016/j.immuni.2015.05.016
125. Hatziioannou A, Alissafi T, Verginis P. Myeloid-Derived Suppressor Cells and T Regulatory Cells in Tumors: Unraveling the Dark Side of the Force. *J Leukoc Biol* (2017) 102:407–21. doi: 10.1189/jlb.5VMR1116-493R
126. Onizuka S, Tawara I, Shimizu J, Sakaguchi S, Fujita T, Nakayama E. Tumor Rejection by In Vivo Administration of Anti-CD25 (Interleukin-2 Receptor Alpha) Monoclonal Antibody. *Cancer Res* (1999) 59:3128–33.
127. Shimizu J, Yamazaki S, Sakaguchi S. Induction of Tumor Immunity by Removing CD25+CD4+ T Cells: A Common Basis Between Tumor Immunity and Autoimmunity. *J Immunol* (1999) 163:5211–8.
128. Fridman WH, Pages F, Sautes-Fridman C, Galon J. The Immune Contexture in Human Tumours: Impact on Clinical Outcome. *Nat Rev Cancer* (2012) 12:298–306. doi: 10.1038/nrc3245
129. Sharabi A, Tsokos MG, Ding Y, Malek TR, Klatzmann D, Tsokos GC. Regulatory T Cells in the Treatment of Disease. *Nat Rev Drug Discov* (2018) 17:823–44. doi: 10.1038/nrd.2018.148
130. Togashi Y, Shitara K, Nishikawa H. Regulatory T Cells in Cancer Immunosuppression - Implications for Anticancer Therapy. *Nat Rev Clin Oncol* (2019) 16:356–71. doi: 10.1038/s41571-019-0175-7
131. Hiraoka N, Onozato K, Kosuge T, Hirohashi S. Prevalence of FOXP3+ Regulatory T Cells Increases During the Progression of Pancreatic Ductal Adenocarcinoma and Its Premalignant Lesions. *Clin Cancer Res* (2006) 12:5423–34. doi: 10.1158/1078-0432.CCR-06-0369
132. Sasada T, Kimura M, Yoshida Y, Kanai M, Takabayashi A. CD4+CD25+ Regulatory T Cells in Patients With Gastrointestinal Malignancies: Possible Involvement of Regulatory T Cells in Disease Progression. *Cancer* (2003) 98:1089–99. doi: 10.1002/cncr.11618
133. Ichihara F, Kono K, Takahashi A, Kawaida H, Sugai H, Fujii H. Increased Populations of Regulatory T Cells in Peripheral Blood and Tumor-Infiltrating Lymphocytes in Patients With Gastric and Esophageal Cancers. *Clin Cancer Res* (2003) 9:4404–8.
134. Liyanage UK, Moore TT, Joo HG, Tanaka Y, Herrmann V, Doherty G, et al. Prevalence of Regulatory T Cells is Increased in Peripheral Blood and Tumor Microenvironment of Patients With Pancreas or Breast Adenocarcinoma. *J Immunol* (2002) 169:2756–61. doi: 10.4049/jimmunol.169.5.2756
135. Hindley JP, Ferreira C, Jones E, Lauder SN, Ladell K, Wynn KK, et al. Analysis of the T-Cell Receptor Repertoires of Tumor-Infiltrating Conventional and Regulatory T Cells Reveals No Evidence for Conversion in Carcinogen-Induced Tumors. *Cancer Res* (2011) 71:736–46. doi: 10.1158/0008-5472.CAN-10-1797
136. Valzasina B, Piconese S, Guiducci C, Colombo MP. Tumor-Induced Expansion of Regulatory T Cells by Conversion of CD4+CD25- Lymphocytes Is Thymus and Proliferation Independent. *Cancer Res* (2006) 66:4488–95. doi: 10.1158/0008-5472.CAN-05-4217
137. Nishikawa H, Kato T, Tanida K, Hiasa A, Tawara I, Ikeda H, et al. CD4+ CD25+ T Cells Responding to Serologically Defined Autoantigens Suppress Antitumor Immune Responses. *Proc Natl Acad Sci USA* (2003) 100:10902–6. doi: 10.1073/pnas.1834479100
138. Adeegbe DO, Liu Y, Lizotte PH, Kamihara Y, Aref AR, Almonte C, et al. Synergistic Immunostimulatory Effects and Therapeutic Benefit of

Combined Histone Deacetylase and Bromodomain Inhibition in Non-Small Cell Lung Cancer. *Cancer Discov* (2017) 7:852–67. doi: 10.1158/2159-8290.CD-16-1020
139. Adeegbe DO, Liu S, Hattersley MM, Bowden M, Zhou CW, Li S, et al. BET Bromodomain Inhibition Cooperates With PD-1 Blockade to Facilitate Antitumor Response in Kras-Mutant Non-Small Cell Lung Cancer. *Cancer Immunol Res* (2018) 6:1234–45. doi: 10.1158/2326-6066.CIR-18-0077
140. Li L, Kim J, Boussiotis VA. IL-1beta-Mediated Signals Preferentially Drive Conversion of Regulatory T Cells But Not Conventional T Cells Into IL-17-Producing Cells. *J Immunol* (2010) 185:4148–53. doi: 10.4049/jimmunol.1001536
141. Li MO, Rudensky AY. T Cell Receptor Signalling in the Control of Regulatory T Cell Differentiation and Function. *Nat Rev Immunol* (2016) 16:220–33. doi: 10.1038/nri.2016.26
142. Lim SA, Wei J, Nguyen TM, Shi H, Su W, Palacios G, et al. Lipid Signalling Enforces Functional Specialization of Treg Cells in Tumours. *Nature* (2021) 591:306–11. doi: 10.1038/s41586-021-03235-6
143. Lowther DE, Goods BA, Lucca LE, Lerner BA, Raddassi K, van Dijk D, et al. PD-1 Marks Dysfunctional Regulatory T Cells in Malignant Gliomas. *JCI Insight* (2016) 1:e85935. doi: 10.1172/jci.insight.85935
144. Wahren-Herlenius M, Dörner T. Immunopathogenic Mechanisms of Systemic Autoimmune Disease. *Lancet* (2013) 382:819–31. doi: 10.1016/S0140-6736(13)60954-X
145. Carbone F, De Rosa V, Carrieri PB, Montella S, Bruzzese D, Porcellini A, et al. Regulatory T Cell Proliferative Potential Is Impaired in Human Autoimmune Disease. *Nat Med* (2014) 20:69–74. doi: 10.1038/nm.3411
146. Dominguez-Villar M, Hafler DA. Regulatory T Cells in Autoimmune Disease. *Nat Immunol* (2018) 19:665–73. doi: 10.1038/s41590-018-0120-4
147. Göschl L, Scheinecker C, Bonelli M. Treg Cells in Autoimmunity: From Identification to Treg-Based Therapies. *Semin Immunopathol* (2019) 41:301–14. doi: 10.1007/s00281-019-00741-8
148. Grant CR, Liberal R, Mieli-Vergani G, Vergani D, Longhi MS. Regulatory T-Cells in Autoimmune Diseases: Challenges, Controversies and–Yet–Unanswered Questions. *Autoimmun Rev* (2015) 14:105–16. doi: 10.1016/j.autrev.2014.10.012
149. Long SA, Buckner JH. CD4+FOXP3+ T Regulatory Cells in Human Autoimmunity: More Than a Numbers Game. *J Immunol* (2011) 187:2061–6. doi: 10.4049/jimmunol.1003224
150. Viglietta V, Baecher-Allan C, Weiner HL, Hafler DA. Loss of Functional Suppression by CD4+CD25+ Regulatory T Cells in Patients With Multiple Sclerosis. *J Exp Med* (2004) 199:971–9. doi: 10.1084/jem.20031579
151. Williams LM, Rudensky AY. Maintenance of the Foxp3-Dependent Developmental Program in Mature Regulatory T Cells Requires Continued Expression of Foxp3. *Nat Immunol* (2007) 8:277–84. doi: 10.1038/ni1437
152. Komatsu N, Mariotti-Ferrandiz ME, Wang Y, Malissen B, Waldmann H, Hori S. Heterogeneity of Natural Foxp3+ T Cells: A Committed Regulatory T-Cell Lineage and an Uncommitted Minor Population Retaining Plasticity. *Proc Natl Acad Sci USA* (2009) 106:1903–8. doi: 10.1073/pnas.0811556106
153. Komatsu N, Okamoto K, Sawa S, Nakashima T, Oh-hora M, Kodama T, et al. Pathogenic Conversion of Foxp3+ T Cells Into TH17 Cells in Autoimmune Arthritis. *Nat Med* (2014) 20:62–8. doi: 10.1038/nm.3432
154. Rubtsov YP, Niec RE, Josefowicz S, Li L, Darce J, Mathis D, et al. Stability of the Regulatory T Cell Lineage In Vivo. *Science* (2010) 329:1667–71. doi: 10.1126/science.1191996
155. Zhou X, Bailey-Bucktrout SL, Jeker LT, Penaranda C, Martínez-Llordella M, Ashby M, et al. Instability of the Transcription Factor Foxp3 Leads to the Generation of Pathogenic Memory T Cells In Vivo. *Nat Immunol* (2009) 10:1000–7. doi: 10.1038/ni.1774
156. Hoffmann P, Boeld TJ, Eder R, Huehn J, Floess S, Wieczorek G, et al. Loss of FOXP3 Expression in Natural Human CD4+CD25+ Regulatory T Cells Upon Repetitive In Vitro Stimulation. *Eur J Immunol* (2009) 39:1088–97. doi: 10.1002/eji.200838904
157. Laurence A, Amarnath S, Mariotti J, Kim YC, Foley J, Eckhaus M, et al. STAT3 Transcription Factor Promotes Instability of Ntreg Cells and Limits Generation of Itreg Cells During Acute Murine Graft-Versus-Host Disease. *Immunity* (2012) 37:209–22. doi: 10.1016/j.immuni.2012.05.027
158. Bailey-Bucktrout SL, Martinez-Llordella M, Zhou X, Anthony B, Rosenthal W, Luche H, et al. Self-Antigen-Driven Activation Induces Instability of Regulatory T Cells During an Inflammatory Autoimmune Response. *Immunity* (2013) 39:949–62. doi: 10.1016/j.immuni.2013.10.016
159. Etesam Z, Nemati M, Ebrahimizadeh MA, Ebrahimi HA, Hajghani H, Khalili T, et al. Altered Expression of Specific Transcription Factors of Th17 (Rorγt, Rorα) and Treg Lymphocytes (FOXP3) by Peripheral Blood Mononuclear Cells From Patients With Multiple Sclerosis. *J Mol Neurosci* (2016) 60:94–101. doi: 10.1007/s12031-016-0789-5
160. Jamshidian A, Shaygannejad V, Pourazar A, Zarkesh-Esfahani SH, Gharagozloo M. Biased Treg/Th17 Balance Away From Regulatory Toward Inflammatory Phenotype in Relapsed Multiple Sclerosis and Its Correlation With Severity of Symptoms. *J Neuroimmunol* (2013) 262:106–12. doi: 10.1016/j.jneuroim.2013.06.007
161. Jones AP, Trend S, Byrne SN, Fabis-Pedrini MJ, Geldenhuys S, Nolan D, et al. Altered Regulatory T-Cell Fractions and Helios Expression in Clinically Isolated Syndrome: Clues to the Development of Multiple Sclerosis. *Clin Transl Immunol* (2017) 6:e143. doi: 10.1038/cti.2017.18
162. Nyirenda MH, Morandi E, Vinkemeier U, Constantin-Teodosiu D, Drinkwater S, Mee M, et al. TLR2 Stimulation Regulates the Balance Between Regulatory T Cell and Th17 Function: A Novel Mechanism of Reduced Regulatory T Cell Function in Multiple Sclerosis. *J Immunol* (2015) 194:5761–74. doi: 10.4049/jimmunol.1400472
163. Dominguez-Villar M, Baecher-Allan CM, Hafler DA. Identification of T Helper Type 1-Like, Foxp3+ Regulatory T Cells in Human Autoimmune Disease. *Nat Med* (2011) 17:673–5. doi: 10.1038/nm.2389
164. 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 Ifnγ+ Th1/Tregs. *Circ Res* (2016) 119:1190–203. doi: 10.1161/CIRCRESAHA.116.309764
165. Arterbery AS, Osafo-Addo A, Avitzur Y, Ciarleglio M, Deng Y, Lobritto SJ, et al. Production of Proinflammatory Cytokines by Monocytes in Liver-Transplanted Recipients With De Novo Autoimmune Hepatitis Is Enhanced and Induces TH1-Like Regulatory T Cells. *J Immunol* (2016) 196:4040–51. doi: 10.4049/jimmunol.1502276
166. Du W, Shen YW, Lee WH, Wang D, Paz S, Kandeel F, et al. Foxp3+ Treg Expanded From Patients With Established Diabetes Reduce Helios Expression While Retaining Normal Function Compared to Healthy Individuals. *PloS One* (2013) 8:e56209. doi: 10.1371/journal.pone.0056209
167. Yamada A, Ushio A, Arakaki R, Tsunematsu T, Kudo Y, Hayashi Y, et al. Impaired Expansion of Regulatory T Cells in a Neonatal Thymectomy-Induced Autoimmune Mouse Model. *Am J Pathol* (2015) 185:2886–97. doi: 10.1016/j.ajpath.2015.07.007
168. Esposito M, Ruffini F, Bergami A, Garzetti L, Borsellino G, Battistini L, et al. IL-17- and IFN-γ-Secreting Foxp3+ T Cells Infiltrate the Target Tissue in Experimental Autoimmunity. *J Immunol* (2010) 185:7467–73. doi: 10.4049/jimmunol.1001519
169. Wang T, Sun X, Zhao J, Zhang J, Zhu H, Li C, et al. Regulatory T Cells in Rheumatoid Arthritis Showed Increased Plasticity Toward Th17 But Retained Suppressive Function in Peripheral Blood. *Ann Rheum Dis* (2015) 74:1293–301. doi: 10.1136/annrheumdis-2013-204228
170. Murdaca G, Tonacci A, Negrini S, Greco M, Borro M, Puppo F, et al. Emerging Role of Vitamin D in Autoimmune Diseases: An Update on Evidence and Therapeutic Implications. *Autoimmun Rev* (2019) 18:102350. doi: 10.1016/j.autrev.2019.102350
171. Erkelens MN, Mebius RE. Retinoic Acid and Immune Homeostasis: A Balancing Act. *Trends Immunol* (2017) 38:168–80. doi: 10.1016/j.it.2016.12.006
172. Lu L, Lan Q, Li Z, Zhou X, Gu J, Li Q, et al. Critical Role of All-Trans Retinoic Acid in Stabilizing Human Natural Regulatory T Cells Under Inflammatory Conditions. *Proc Natl Acad Sci USA* (2014) 111:E3432–40. doi: 10.1073/pnas.1408780111
173. Lu L, Ma J, Li Z, Lan Q, Chen M, Liu Y, et al. All-Trans Retinoic Acid Promotes TGF-β-Induced Tregs via Histone Modification But Not DNA Demethylation on Foxp3 Gene Locus. *PloS One* (2011) 6:e24590. doi: 10.1371/journal.pone.0024590
174. Jeffery LE, Burke F, Mura M, Zheng Y, Qureshi OS, Hewison M, et al. 1,25-Dihydroxyvitamin D3 and IL-2 Combine to Inhibit T Cell Production of Inflammatory Cytokines and Promote Development of Regulatory T Cells

Expressing CTLA-4 and Foxp3. *J Immunol* (2009) 183:5458–67. doi: 10.4049/jimmunol.0803217
175. Kang SW, Kim SH, Lee N, Lee WW, Hwang KA, Shin MS, et al. 1,25-Dihyroxyvitamin D3 Promotes FOXP3 Expression via Binding to Vitamin D Response Elements in Its Conserved Noncoding Sequence Region. *J Immunol* (2012) 188:5276–82. doi: 10.4049/jimmunol.1101211
176. Baban B, Chandler PR, Sharma MD, Pihkala J, Koni PA, Munn DH, et al. IDO Activates Regulatory T Cells and Blocks Their Conversion Into Th17-Like T Cells. *J Immunol* (2009) 183:2475–83. doi: 10.4049/jimmunol.0900986
177. Brown J, Robusto B, Morel L. Intestinal Dysbiosis and Tryptophan Metabolism in Autoimmunity. *Front Immunol* (2020) 11:1741–83. doi: 10.3389/fimmu.2020.01741
178. Shi LZ, Wang R, Huang G, Vogel P, Neale G, Green DR, et al. HIF1alpha-Dependent Glycolytic Pathway Orchestrates a Metabolic Checkpoint for the Differentiation of TH17 and Treg Cells. *J Exp Med* (2011) 208:1367–76. doi: 10.1084/jem.20110278
179. Rathmell JC, Elstrom RL, Cinalli RM, Thompson CB. Activated Akt Promotes Increased Resting T Cell Size, CD28-Independent T Cell Growth, and Development of Autoimmunity and Lymphoma. *Eur J Immunol* (2003) 33:2223–32. doi: 10.1002/eji.200324048
180. Patel RK, Mohan C. PI3K/AKT Signaling and Systemic Autoimmunity. *Immunol Res* (2005) 31:47–55. doi: 10.1385/IR:31:1:47
181. Martins F, Sofiya L, Sykiotis GP, Lamine F, Maillard M, Fraga M, et al. Adverse Effects of Immune-Checkpoint Inhibitors: Epidemiology, Management and Surveillance. *Nat Rev Clin Oncol* (2019) 16:563–80. doi: 10.1038/s41571-019-0218-0
182. Rech AJ, Mick R, Martin S, Recio A, Aqui NA, Powell DJJr., et al. CD25 Blockade Depletes and Selectively Reprograms Regulatory T Cells in Concert With Immunotherapy in Cancer Patients. *Sci Transl Med* (2012) 4:134ra62. doi: 10.1126/scitranslmed.3003330
183. Mahnke K, Schonfeld K, Fondel S, Ring S, Karakhanova S, Wiedemeyer K, et al. Depletion of CD4+CD25+ Human Regulatory T Cells In Vivo: Kinetics of Treg Depletion and Alterations in Immune Functions *In Vivo* and *In Vitro*. *Int J Cancer* (2007) 120:2723–33. doi: 10.1002/ijc.22617
184. Kachikwu EL, Iwamoto KS, Liao YP, DeMarco JJ, Agazaryan N, Economou JS, et al. Radiation Enhances Regulatory T Cell Representation. *Int J Radiat Oncol Biol Phys* (2011) 81:1128–35. doi: 10.1016/j.ijrobp.2010.09.034
185. Smilowitz HM, Sasso D, Lee EW, Goh G, Micca PL, Dilmanian FA. Therapy Model for Advanced Intracerebral B16 Mouse Melanoma Using Radiation Therapy Combined With Immunotherapy. *Cancer Immunol Immunother* (2013) 62:1187–97. doi: 10.1007/s00262-013-1423-9
186. Jacobs JF, Punt CJ, Lesterhuis WJ, Sutmuller RP, Brouwer HM, Scharenborg NM, et al. Dendritic Cell Vaccination in Combination With Anti-CD25 Monoclonal Antibody Treatment: A Phase I/II Study in Metastatic Melanoma Patients. *Clin Cancer Res* (2010) 16:5067–78. doi: 10.1158/1078-0432.CCR-10-1757
187. Solomon I, Amann M, Goubier A, Vargas FA, Zervas D, Qing C, et al. CD25-Treg-Depleting Antibodies Preserving IL-2 Signaling on Effector T Cells Enhance Effector Activation and Antitumor Immunity. *Nat Cancer* (2020) 1:1153–66. doi: 10.1038/s43018-020-00133-0
188. Saleh R, Elkord E. Treg-Mediated Acquired Resistance to Immune Checkpoint Inhibitors. *Cancer Lett* (2019) 457:168–79. doi: 10.1016/j.canlet.2019.05.003
189. Le Mercier I, Chen W, Lines JL, Day M, Li J, Sergent P, et al. VISTA Regulates the Development of Protective Antitumor Immunity. *Cancer Res* (2014) 74:1933–44. doi: 10.1158/0008-5472.CAN-13-1506
190. 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
191. Marangoni F, Zhakyp A, Corsini M, Geels SN, Carrizosa E, Thelen M, et al. Expansion of Tumor-Associated Treg Cells Upon Disruption of a CTLA-4-Dependent Feedback Loop. *Cell* (2021) 184(15):3998–4015.e19. doi: 10.1016/j.cell.2021.05.027
192. Selby MJ, Engelhardt JJ, Quigley M, Henning KA, Chen T, Srinivasan M, et al. Anti-CTLA-4 Antibodies of IgG2a Isotype Enhance Antitumor Activity Through Reduction of Intratumoral Regulatory T Cells. *Cancer Immunol Res* (2013) 1:32–42. doi: 10.1158/2326-6066.CIR-13-0013
193. Simpson TR, Li F, Montalvo-Ortiz W, Sepulveda MA, Bergerhoff K, Arce F, et al. Fc-Dependent Depletion of Tumor-Infiltrating Regulatory T Cells Co-Defines the Efficacy of Anti-CTLA-4 Therapy Against Melanoma. *J Exp Med* (2013) 210:1695–710. doi: 10.1084/jem.20130579
194. Arce Vargas F, Furness AJS, Litchfield K, Joshi K, Rosenthal R, Ghorani E, et al. Fc Effector Function Contributes to the Activity of Human Anti-CTLA-4 Antibodies. *Cancer Cell* (2018) 33(4):649–63.e4. doi: 10.1016/j.ccell.2018.02.010
195. Sharma A, Subudhi SK, Blando J, Vence L, Wargo J, Allison JP, et al. Anti-CTLA-4 Immunotherapy Does Not Deplete FOXP3(+) Regulatory T Cells (Tregs) in Human Cancers-Response. *Clin Cancer Res* (2019) 25:3469–70. doi: 10.1158/1078-0432.CCR-19-0402
196. Zhang B, Chikuma S, Hori S, Fagarasan S, Honjo T. Nonoverlapping Roles of PD-1 and FoxP3 in Maintaining Immune Tolerance in a Novel Autoimmune Pancreatitis Mouse Model. *Proc Natl Acad Sci USA* (2016) 113:8490–5. doi: 10.1073/pnas.1608873113
197. Ko K, Yamazaki S, Nakamura K, Nishioka T, Hirota K, Yamaguchi T, et al. Treatment of Advanced Tumors With Agonistic Anti-GITR mAb and its Effects on Tumor-Infiltrating Foxp3+CD25+CD4+ Regulatory T Cells. *J Exp Med* (2005) 202:885–91. doi: 10.1084/jem.20050940
198. Schoenhals JE, Cushman TR, Barsoumian HB, Li A, Cadena AP, Niknam S, et al. Anti-Glucocorticoid-Induced Tumor Necrosis Factor-Related Protein (GITR) Therapy Overcomes Radiation-Induced Treg Immunosuppression and Drives Abscopal Effects. *Front Immunol* (2018) 9:2179–92. doi: 10.3389/fimmu.2018.02170
199. Turk MJ, Guevara-Patino JA, Rizzuto GA, Engelhorn ME, Sakaguchi S, Houghton AN. Concomitant Tumor Immunity to a Poorly Immunogenic Melanoma Is Prevented by Regulatory T Cells. *J Exp Med* (2004) 200:771–82. doi: 10.1084/jem.20041130
200. Bulliard Y, Jolicoeur R, Zhang J, Dranoff G, Wilson NS, Brogdon JL. OX40 Engagement Depletes Intratumoral Tregs via Activating FcgammaRs, Leading to Antitumor Efficacy. *Immunol Cell Biol* (2014) 92:475–80. doi: 10.1038/icb.2014.26
201. Curti BD, Kovacsovics-Bankowski M, Morris N, Walker E, Chisholm L, Floyd K, et al. OX40 is a Potent Immune-Stimulating Target in Late-Stage Cancer Patients. *Cancer Res* (2013) 73:7189–98. doi: 10.1158/0008-5472.CAN-12-4174
202. Faget J, Bendriss-Vermare N, Gobert M, Durand I, Olive D, Biota C, et al. ICOS-Ligand Expression on Plasmacytoid Dendritic Cells Supports Breast Cancer Progression by Promoting the Accumulation of Immunosuppressive CD4+ T Cells. *Cancer Res* (2012) 72:6130–41. doi: 10.1158/0008-5472.CAN-12-2409
203. Zappasodi R, Sirard C, Li Y, Budhu S, Abu-Akeel M, Liu C, et al. Rational Design of Anti-GITR-Based Combination Immunotherapy. *Nat Med* (2019) 25:759–66. doi: 10.1038/s41591-019-0420-8
204. Ali K, Soond DR, Pineiro R, Hagemann T, Pearce W, Lim EL, et al. Inactivation of PI(3)K P110delta Breaks Regulatory T-Cell-Mediated Immune Tolerance to Cancer. *Nature* (2014) 510:407–11. doi: 10.1038/nature13444
205. Ohta A, Sitkovsky M. Extracellular Adenosine-Mediated Modulation of Regulatory T Cells. *Front Immunol* (2014) 5:304. doi: 10.3389/fimmu.2014.00304
206. Tada Y, Togashi Y, Kotani D, Kuwata T, Sato E, Kawazoe A, et al. Targeting VEGFR2 With Ramucirumab Strongly Impacts Effector/Activated Regulatory T Cells and CD8(+) T Cells in the Tumor Microenvironment. *J Immunother Cancer* (2018) 6:106. doi: 10.1186/s40425-018-0403-1
207. Terme M, Pernot S, Marcheteau E, Sandoval F, Benhamouda N, Colussi O, et al. VEGFA-VEGFR Pathway Blockade Inhibits Tumor-Induced Regulatory T-Cell Proliferation in Colorectal Cancer. *Cancer Res* (2013) 73:539–49. doi: 10.1158/0008-5472.CAN-12-2325
208. Holmgaard RB, Schaer DA, Li Y, Castaneda SP, Murphy MY, Xu X, et al. Targeting the TGFbeta Pathway With Galunisertib, a TGFbetaRI Small Molecule Inhibitor, Promotes Anti-Tumor Immunity Leading to Durable, Complete Responses, as Monotherapy and in Combination With Checkpoint Blockade. *J Immunother Cancer* (2018) 6:47. doi: 10.1186/s40425-018-0356-4
209. Lan Y, Zhang D, Xu C, Hance KW, Marelli B, Qi J, et al. Enhanced Preclinical Antitumor Activity of M7824, a Bifunctional Fusion Protein Simultaneously

Targeting PD-L1 and TGF-Beta. *Sci Transl Med* (2018) 10:eaan5488. doi: 10.1126/scitranslmed.aan5488

210. Zappasodi R, Serganova I, Cohen IJ, Maeda M, Shindo M, Senbabaoglu Y, et al. CTLA-4 Blockade Drives Loss of Treg Stability in Glycolysis-Low Tumours. *Nature* (2021) 591:652–8. doi: 10.1038/s41586-021-03326-4
211. Cohen AD, Schaer DA, Liu C, Li Y, Hirschhorn-Cymmerman D, Kim SC, et al. Agonist Anti-GITR Monoclonal Antibody Induces Melanoma Tumor Immunity in Mice by Altering Regulatory T Cell Stability and Intra-Tumor Accumulation. *PloS One* (2010) 5:e10436. doi: 10.1371/journal.pone.0010436
212. Schaer DA, Budhu S, Liu C, Bryson C, Malandro N, Cohen A, et al. GITR Pathway Activation Abrogates Tumor Immune Suppression Through Loss of Regulatory T Cell Lineage Stability. *Cancer Immunol Res* (2013) 1:320–31. doi: 10.1158/2326-6066.CIR-13-0086
213. Grigoriou M, Banos A, Hatzioannou A, Kloetgen A, Kouzis P, Aggouraki D, et al. Regulatory T-Cell Transcriptomic Reprogramming Characterizes Adverse Events by Checkpoint Inhibitors in Solid Tumors. *Cancer Immunol Res* (2021) 9:726–34. doi: 10.1158/2326-6066.CIR-20-0969

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.

frontiers in Immunology

ORIGINAL RESEARCH
published: 19 November 2021
doi: 10.3389/fimmu.2021.718359

Cellular and Molecular Phenotypes of pConsensus Peptide (pCons) Induced CD8$^+$ and CD4$^+$ Regulatory T Cells in Lupus

Ram P. Singh[1,2*†], *Bevra H. Hahn*[2,3] *and David S. Bischoff*[1,3]

[1] Research Service, Veteran Administration Greater Los Angeles Healthcare System, Los Angeles, CA, United States,
[2] Division of Rheumatology, Department of Medicine, University of California, Los Angeles, Los Angeles, CA, United States,
[3] Department of Medicine, University of California, Los Angeles, Los Angeles, CA, United States

OPEN ACCESS

Edited by:
Ciriaco A Piccirillo, McGill University, Canada

Reviewed by:
Bergithe Eikeland Oftedal, University of Bergen, Norway
Aijing Liu, Second Hospital of Hebei Medical University, China

***Correspondence:**
Ram P. Singh
ram.singh@va.gov;
rpsingh11@hotmail.com

†Present Address:
Ram P. Singh, Research Service, Veteran Administration Greater Los Angeles Healthcare System, Los Angeles, CA, United States

Specialty section:
This article was submitted to Immunological Tolerance and Regulation, a section of the journal Frontiers in Immunology

Received: *31 May 2021*
Accepted: *26 October 2021*
Published: *19 November 2021*

Citation:
Singh RP, Hahn BH and Bischoff DS (2021) Cellular and Molecular Phenotypes of pConsensus Peptide (pCons) Induced CD8$^+$ and CD4$^+$ Regulatory T Cells in Lupus. Front. Immunol. 12:718359. doi: 10.3389/fimmu.2021.718359

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease with widespread inflammation, immune dysregulation, and is associated with the generation of destructive anti-DNA autoantibodies. We have shown previously the immune modulatory properties of pCons peptide in the induction of both CD4$^+$ and CD8$^+$ regulatory T cells which can in turn suppress development of the autoimmune disease in (NZB/NZW) F1 (BWF1) mice, an established model of lupus. In the present study, we add novel protein information and further demonstrate the molecular and cellular phenotypes of pCons-induced CD4$^+$ and CD8$^+$ T_{reg} subsets. Flow cytometry analyses revealed that pCons induced CD8$^+$ T_{reg} cells with the following cell surface molecules: CD25^{high}CD28$^{high\ and\ low}$ subsets (shown earlier), CD62L^{high}, CD122^{low}, PD1^{low}, CTLA4^{low}, CCR7^{low} and 41BB^{high}. Quantitative real-time PCR (qRT-PCR) gene expression analyses revealed that pCons-induced CD8$^+$ T_{reg} cells downregulated the following several genes: Regulator of G protein signaling (*RGS2), RGS16, RGS17*, *BAX, GPT2, PDE3b, GADD45β and* programmed cell death *1 (PD1).* Further, we confirmed the down regulation of these genes by Western blot analyses at the protein level. To our translational significance, we showed herein that pCons significantly increased the percentage of CD8$^+$FoxP3$^+$ T cells and further increased the mean fluorescence intensity (MFI) of FoxP3 when healthy peripheral blood mononuclear cells (PBMCs) are treated with pCons (10 μg/ml, for 24-48 hours). In addition, we found that pCons reduced apoptosis in CD4$^+$ and CD8$^+$ T cells and B220$^+$ B cells of BWF1 lupus mice. These data suggest that pCons stimulates cellular, immunological, and molecular changes in regulatory T cells which in turn protect against SLE autoimmunity.

Keywords: pCons, regulatory T cells, systemic lupus erythematosus, anti-DNA Ab, immune tolerance, immune regulation

INTRODUCTION

SLE is an autoimmune disease characterized by widespread inflammation, autoantibody production, and immune complex deposition. Regulatory T cells (T_{reg}) are protective in many inflammatory and autoimmune diseases including SLE. The modulation of abnormal immune regulation is an object of intense investigation in autoimmune diseases. A therapeutic goal is to limit

the number and activity of abnormal pathogenic cells and autoantibodies through restoration of immune system self-tolerance. One way to achieve that is by administrating peptides (such as pConsensus peptide, edratide and nucleosomal peptides) that induce regulatory T cells (1–11). Another approach used recently utilized nanoparticles for expanding regulatory T cells to treat autoimmune diseases including lupus (12–15). Whereas a decrease in the number and/or function of regulatory $CD4^+$ T cells has been extensively studied in SLE (16–24), the role and characterization of the $CD8^+$ T_{reg} subset is less clear. Investigating the genes, regulatory networks, and signaling pathways that regulate the functional activity and survival of $CD8^+$ T_{reg} cells is important for development of therapies for restoring immune homeostasis in SLE and other autoimmune diseases. However, in order to rationally intervene to restore immune homeostasis, there is much that remains to be understood about the molecular phenotypes, mechanisms and pathways that govern the differentiation, expansion, maintenance, and regulatory function of $CD8^+$ T_{reg}. We have developed a unique model in which $CD8^+$ regulatory T cells can be induced to suppress the development of autoimmune disease in an animal model of lupus, the (NZB/NZW) F1 (BWF1) mouse (3, 5, 25, 26). In this model, we have demonstrated that synthetic peptides (pCons) based on T cell determinants in the VH region of IgG which encode murine antibodies to DNA that bind to MHC Class I/II regions can activate $CD8^+$ T cells *in vitro*, which can result in the suppression of co-cultured $CD4^+$ T helper cells and B cell activities (26, 27). In addition, when pCons is administered *in vivo*, we can demonstrate the suppression of anti-DNA antibody production, and subsequent nephritis. However, the cellular and molecular phenotypes of pCons-induced $CD8^+$ regulatory T cells are not yet completely clear. In this study, we have further provided novel information and defined the immunological and molecular phenotypes of pCons-induced $CD8^+$ T regulatory cells and $CD4^+$ regulatory T cells. We also showed that pCons treatment reduces apoptosis in $CD4^+$ T cells, and $CD8^+$ T cells and $B220^+$ B cells. To the translational significance, we showed herein that pCons also induces $CD8^+FoxP3^+$ T_{reg} cells in healthy human peripheral blood mononuclear cells (PBMCs).

MATERIALS AND METHODS

Mice

NZB (H-2d/d), NZW (H-2z/z) and NZB/NZW F1 (H-2d/z) mice were purchased from the Jackson Laboratories (Bar Harbor, ME, USA) or bred at the University of California Los Angeles (UCLA). All mice were treated in accordance with the guidelines of the University of California Los Angeles Animal Research Committee, an Institution accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). Mice were housed in pathogen-free conditions according to the National Institutes of Health (NIH) guidelines for the use of experimental animals. Female mice were used for all experiments.

Subjects

We enrolled 6 healthy female donors (19-70 years of age) with no history of autoimmune disease. Subjects had regular menstrual cycles and were not taking any contraceptives or sex hormones. Written informed consent was obtained from each subject who participated in the study. The study was approved by the Institutional Review Board (# 11-000907) of the University of California Los Angeles.

Peptides

The pCons peptide used in this study and the MHC molecules they bind have been described earlier (26, 28). pCons (FIEWNKLRFRQGLEW), the artificial tolerizing peptide, contains T-cell determinants based on the J558 VH regions of several murine anti-dsDNA Ab from BWF1 mice (3, 5, 23, 26, 27, 29). Peptides were synthesized at Chiron Biochemicals (San Diego, CA, USA), purified to a single peak on high-performance liquid chromatography, and analyzed by mass spectroscopy for expected amino acid content.

Treatment of Mice

Ten- to twelve-week-old female BWF1 mice received a single i.v. dose of 1 mg of pCons, dissolved in saline, as reported previously (26, 27, 30) for tolerance induction. For immunophenotyping of regulatory T cells, female BWF1 mice were used and injected with pCons. Control mice received either a similar amount of pNeg (negative control peptide) or saline.

Cell Isolation, Preparation, Immunophenotyping, and Flow Cytometry

Spleen cells were isolated ~1 week after administration of the pCons peptide from tolerized, saline-treated, or naïve BWF1 mice. Single cell suspensions of splenocytes were prepared by passing cells through cell strainers (40μm) (Fisher). ACK lysing buffer, (Sigma, St Louis, MO, USA) was used to lyse red blood cells. Cells were washed and re-suspended in RPMI complete media. RPMI 1640-complete media was supplemented with L-glutamine (2 mM), penicillin (100 units/ml), streptomycin (0.1 mg/ml), 2-mercaptoethanol (Gibco) and 10% fetal bovine serum (FBS). FACS staining buffer was obtained from eBiosciences, BD Pharmingen, and/or BioLegend Inc. Cell subsets were further enriched following incubation with anti-CD4 (L3T4), anti-B (CD45R/B220), anti-CD8 (CD8a Ly-2), and microbeads from Miltenyi Biotech (Auburn, CA, USA). Purity of cells was determined to be more than 90% pure as assessed by flow cytometry (FACS). For immunophenotyping, isolated cells were washed with FACS buffer and 1–2 million cells were used for cell surface staining. Before staining, cells were incubated with rat anti-mouse CD16/CD32 (FC III/II receptor) Ab to block nonspecific binding.

For regulatory T cell immunophenotyping, spleen cells were stained with CD4 (L3T4), (RPA-T4), CD8 (Ly-2), CD25 (PC61), CD28 (37.51), CD62L (MEL-14), CD122 (TM-β1), PD1(RMP1), CCR7(4B12), GITR (DTA-1, AITR, TNFRSF18), CTLA-4

(UC10-4F10-11) and 4IBB(1AH2) antibodies for FACS analysis. Antibodies for cell surface staining and isotype controls were from BD Biosciences, BD Pharmingen, eBiosciences, or BioLegend. FoxP3 (PCH101) staining was performed with an eBiosciences intracellular kit (Cat #12-4776). Before intracellular FoxP3 staining, cells were stained with cell surface molecules (CD4, CD8, CD25, CD28, CD62L, CD122) as per manufacturer's protocol. Cells were fixed and permeabilized, washed with permeabilization buffer, and then stained with anti-human FoxP3 (PCH101) antibody in 1X permeabilization buffer (eBiosciences), washed again with permeabilization buffer, and then the samples analyzed by FACS at the UCLA flow Core facility. Data was collected using an FACSCalibur (BD Biosciences) and analyzed with BD Cell Quest software (Becton-Dickinson, Mountain View, CA) or *De Novo* FCS Express Ver. 7 software (Ontario, Canada).

Human Peripheral Blood Mononuclear Cells (PBMCs) Isolation and Preparation

For human studies, peripheral blood mononuclear cells (PBMCs) were isolated on a density gradient (Histopaque-1077, Sigma-Aldrich, St. Louis, MO, USA) from blood samples of healthy volunteers. Lymphocytes were washed twice in RPMI complete media. Red blood cells (RBC) were lysed with RBC lysing solution (Sigma-Aldrich, St. Louis, MO, USA). After washing cells were stained with fluorochrome -labeled monoclonal antibodies (mAbs) and analyzed by FACS.

Western Blot Analysis

Western blot analyses were performed as described earlier (31). In brief, cell lysates were prepared from the $CD8^+$ T cells of naïve and pCons-treated BWF1 mice. Cells were lysed with RIPA buffer (150 nM NaCl, 1.0% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 10 mM Tris, pH 7.3) supplemented with Protease Arrest protease inhibitor cocktail solution (G Biosciences, Maryland Heights, MO, USA). Protein was measured from each sample using the Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA) and an equal amount of protein was loaded in each well. The lysates were resolved on a 4–12% NuPage gel (Invitrogen, Carlsbad, CA, USA) under reducing conditions. Proteins were electro-transferred onto a polyvinylidene fluoride membrane (Invitrogen). The membranes were blocked with 3% BSA and immunoblotted with a protein-specific antibodies [GPT2 (ab80947), Abcam; PD1 (DO-1), sc-126 Santa Cruz Biotechnology, Inc; PD1 (ab58811) Abcam; GADD45b (K-12), sc-133606, Santa Cruz Biotechnologies, Inc; p53 (DO-1) sc-126, Santa Cruz Biotechnologies, Inc, Santa Cruz, CA, USA, (1: 200 - 1:1000 dilution range); Bax (1:1000 dilution) Cat # #2772, Cell Signaling Technology, Danvers, MA; PDE3b, H-300, sc-20793 (1:1000 dilution); RGS16 (H-100), sc-30218 (1:1000 dilution) or β-actin (1:100 000 dilution; Sigma, Inc]. Following washing, the membranes were incubated in secondary antibodies (1:2500 dilution; Santa Cruz Inc, Santa Cruz, CA, USA). All blocking, incubation and washing steps were performed in TBST (TBS and 0.1% Tween-20). Proteins were visualized using ECL (GE Healthcare, Buckinghamshire, UK).

RNA Isolation and Real-Time PCR

Total cellular RNA was isolated from purified cell subsets from saline-treated or pCons-tolerized BWF1 mice with TRIzol (Invitrogen, Carlsbad, CA, USA) as per manufacturer's protocols. One-step-real time PCR was analyzed as described earlier (3, 5, 26, 29). Each experimental group consists of the pooled spleen cells of 3–4 mice from each group, naïve $CD8^+$ T cells or tolerized $CD8^+$ T cells. One-step RT-PCR was performed (Applied Biosystems, Foster City, CA, USA) using 100 ng of total RNA. Quantitative real-time reverse transcription was performed using TaqMan technology on an ABI Prism 7900 HT Sequence Detection System (Applied Biosystems). Primers and probes of regulator of G protein signaling genes (RGS2, RGS16, and RGS17), glutamic pyruvate transaminase 2 (GPT2), BAX (Bcl-2-associated X protein), programmed cell death-1 (PD1), growth arrest and DNA damage inducible 45 beta (GADD45β), and phosphodiesterase 3b (PDE3b), and GAPDH were obtained from Applied Biosystems (Foster City, CA, USA). The other oligonucleotide sequences used for the primers and TaqMan probes (Applied Biosystem, Foster City, CA) are described (3, 5, 26, 29). GAPDH was used as an endogenous control in each experimental set.

Measurement of Apoptosis

Assays were performed to measure apoptosis as described earlier (5, 26). In brief, splenocytes were obtained from both naïve and pCons-treated BWF1 mice. RBC were lysed, cells washed, and stained with fluorochrome-labeled specific antibodies [CD4 (PerCP), CD8 (PE), B220 (APC), and Annexin V (FITC)] and flow cytometry performed.

Statistical Analyses

Data was analyzed using GraphPad Prism 4.0 Software (San Diego, CA). Comparisons between the two groups were performed using paired one- or two-tailed Student's *t*-test. Nonparametric testing among more than two groups was performed by one-way ANOVA. Results are expressed as mean ± SEM. $p<0.05$ was considered significant.

RESULTS

pCons Treatment Alters Cell Surface Expression of CD25, CD122, and Increased Intracellular FoxP3 Expression in $CD4^+$ T Cells and Further Modifies Cell Surface Expression of CD25, CD28, CTLA-4 and 41BB in $CD8^+$ T Cells in BWF1 Mice

To explore the tolerogenic immune responses of pCons peptide in the present study, we determined the various cell surface expression markers by flow cytometry in both $CD4^+$ and $CD8^+$ T cells in pCons-treated and negative control peptide and or saline-treated BWF1 mice. We found that pCons treatment increased the cell surface expression of CD25 and CD122 in $CD4^+$ T cells compared to naïve $CD4^+$ T cells (**Figure 1A Panels A, C, E, H, J, L**). We also found that pCons treatment increased the

intracellular FoxP3 expression in pCons-treated CD4$^+$ T cells (**Figure 1A Panels B, D, F, G, I, K**). Next, we investigated the effect of pCons on CD8$^+$ T cells. Our data demonstrate that pCons treatment modified the cell surface expression of CD25 (increased), CD28 (increased), CTLA-4 (decreased) and 41BB (no changed) in CD8$^+$ T cells (**Figure 1B Panels A–L**). Gating strategy is shown in (**Supplementary Figure 1A** and **Figure 1B** Panels A–H). Previously, we have demonstrated that pCons treatment increased the FoxP3 expression in CD8$^+$ T cells (3, 26). In this study, we re-validated our previous findings of FoxP3 and added novel information for additional cell surface phenotypes including FoxP3 with cumulative data of 4-6

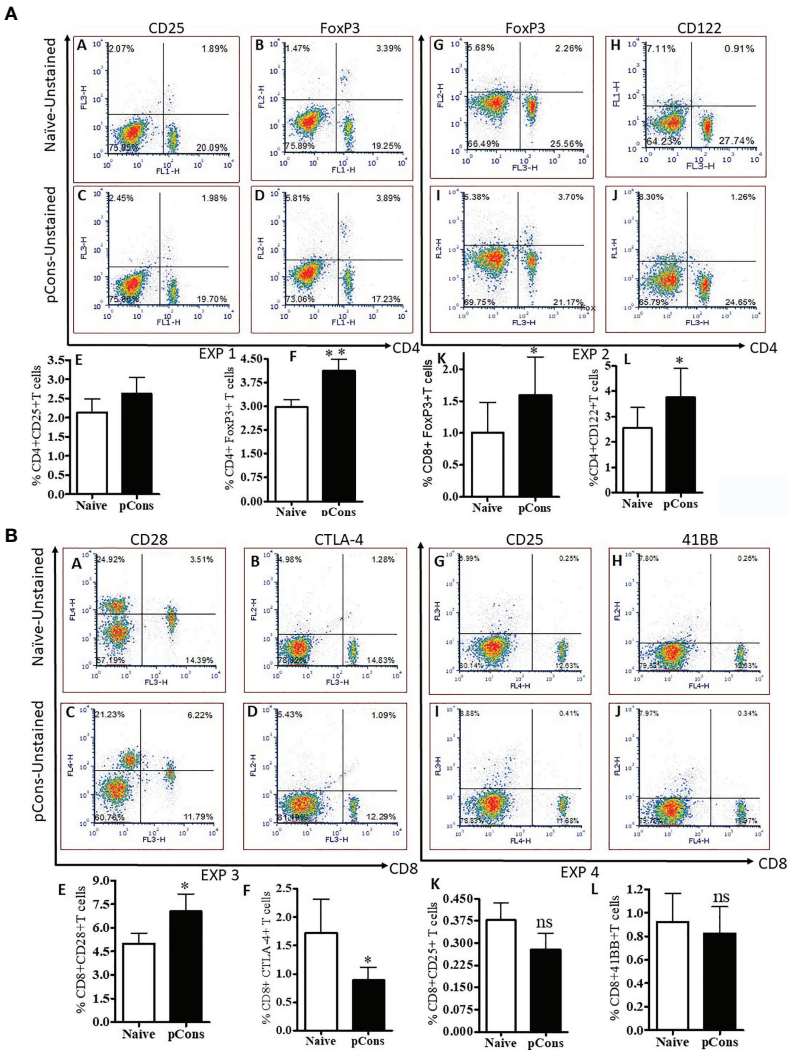


FIGURE 1 | pCons treatment alters cell surface expression of CD25, CD122, and increased intracellular FoxP3 expression in CD4$^+$ T cells and further modifies cell surface expression of CD25, CD28, CTLA-4 and 41BB in CD8$^+$ T cells in BWF1 mice. Female 10-12-wk old BWF1 mice were treated with pCons (1 mg *i.v.*). After one-two week treatment, splenocytes were obtained both from naive and pCons-treated BWF1 mice. RBC were lysed, cells washed, and stained with fluorochrome-labeled specific antibodies (CD4, CD8, CD25, CD28, CD122, CTLA-4, and 41BB). FACS analysis was performed on a FACSCalibur^{TM} with cell Quest^{TM} software (BD Biosciences, San Jose, CA) and analyzed using *De Novo* FCS Express software (Ontario, Canada). Intracellular FoxP3 expression was analyzed after cell fixation and permeabilization as per manufacturer's protocol (eBiosciences, San Diego, CA, USA). **(A)** CD4$^+$ T cells, and **(B)** CD8$^+$ T cells data, two experiments each. **(A)** Exp 1. Panel (A) Naïve CD4$^+$CD25$^+$ T cells; (B) Naïve CD4$^+$FoxP3$^+$ T cells; (C) pCons CD4$^+$CD25$^+$ T cells; (D) pCons CD4$^+$FoxP3$^+$ T cells. (E) Cumulative data of CD4$^+$CD25$^+$T cells (4-5 experiments of two/three mice); (F) Cumulative data of CD4$^+$FoxP3$^+$ T cells (4-5 experiments of two/three mice). Exp 2. (G) Naïve CD4$^+$FoxP3$^+$ T cells; (H) Naïve CD4$^+$CD122$^+$ T cells; (I) pCons CD4$^+$FoxP3$^+$ T cells., (J) pCons CD4$^+$CD122$^+$ T cells. (K) Cumulative data (6 experiments) of CD8$^+$FoxP3$^+$ T cells. (L) Cumulative data of CD4$^+$CD122$^+$ T cells (4 experiments of two/three mice). **(B)** Exp 3. (A) Naïve CD8$^+$CD28$^+$ T cells; (B) Naïve CD8$^+$CTLA-4$^+$ T cells; (C) pCons CD8$^+$CD28$^+$ T cells; (D) pCons CD8$^+$CTLA-4$^+$ T cells. (E) Cumulative data of CD8$^+$CD28$^+$ T cells (5-6 experiments). (F) Cumulative data of CD8$^+$CTLA-4$^+$ T cells (5-6 experiments). Exp.4. (G) Naïve CD8$^+$CD25$^+$ T cells; (H) Naïve CD8$^+$41BB$^+$ T cells, (I) pCons CD8$^+$CD25$^+$ T cells; (J) pCons CD8$^+$41BB$^+$ T cells. (K) Cumulative (6-7 experiments data) of CD8$^+$CD25$^+$ T cells, (L) Cumulative (4 experiments data) of CD8$^+$41BB$^+$ T cells. Minimum 10,000 cells were gated, and only live cells were used for data analyses. Dead cells were excluded from the analyses. *$p < 0.05$. **$p < 0.001$. ns, not significant.

experiments (**Figure1A Panel K**). Altogether, these data indicate that pCons treatment induced the various cell surface markers including intracellular FoxP3 in both $CD4^+$ and $CD8^+$T cells.

pCons Treatment Induces and Modifies Cell Surface Expression of CD62L, CD122, and CCR7 in $CD8^+$ T Cells in BWF1 Mice

Previously we showed that pCons treatment increased the number of regulatory $CD4^+$ and $CD8^+$ T cells and modulated their functions including the ability for the suppression of anti-DNA antibody in BWF1 mice (3, 5, 23, 26). In the present study, we were interested to see whether pCons treatment of BWF1 mice changes the cell surface expression of CD122, CD62L, PD1, and CCR7 in $CD8^+$ T cells since these markers have been implicated in the regulatory phenotypes of $CD8^+$ T cells (32–36). We found two-fold increase in cell surface expression of CD62L and further increase in percent expression of $CD8^+$ $CD62L^+$ T cells and a decrease in the CD122 and CCR7 mean fluorescence intensity in pCons-treated $CD8^+$ T cells compared to negative control peptide or saline-treated $CD8^+$ T cells (**Figures 2A–E**). PD1 cell surface expression was decreased in pCons-treated $CD8^+$ T cells (**Figure 2D**). Further, Western blot analysis demonstrated that protein levels of CCR7 are decreased in $CD8^+$ T cells after pCons tolerance (**Figure 2F**). Taken together, these data suggest that pCons treatment has differential immune-regulatory effects on $CD8^+$ T cells and on CD62L, CD122, PD1, and CCR7.

pCons Treatment Modifies Expression of Regulator of G-Protein Signaling Genes (RGS2, RGS16, RGS17), Interferon-Induced, and Apoptotic Genes in $CD8^+$ T Cells

To determine whether there is cross-regulation of regulator of G protein signaling and interferon genes and whether pCons affect this cross-regulation in $CD8^+$ T cells, we tested the expression of RGS and IFNs genes in pCons-treated $CD8^+$ T cells. We have shown previously that pCons-induced $CD8^+$ T regulatory cells are genetically reprogrammed following pCons induction (26, 29, 31). These pCons-induced $CD8^+$ T_{reg} cells display i) resist to apoptosis; ii) have immunosuppressive programs; and iii) traffic to sites of inflammation to inhibit the development of autoantibody formation. Our previous gene chip array analyses demonstrated that pCons-induced $CD8^+$ T_{reg} cells have upregulated genes including; interferon inducible *202b (Ifi202b), FoxP3, Bcl2*, transformation related protein *53 (TP53)*

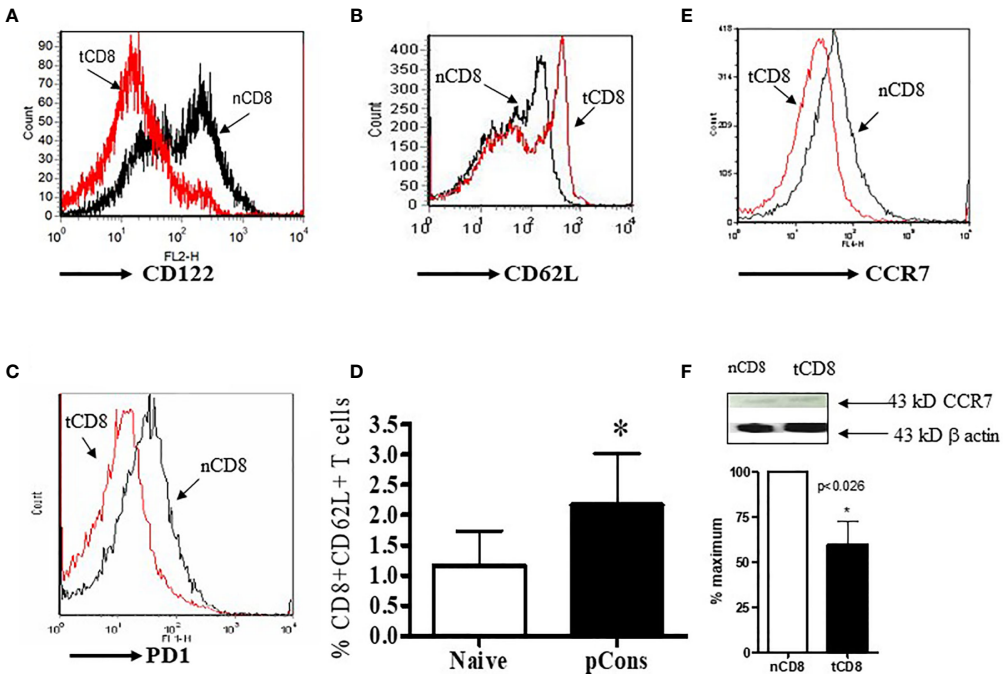


FIGURE 2 | pCons treatment modified the cell surface expression of CD122, CD62L, CCR7, and PD1 in $CD8^+$ T cells in BWF1 mice. Female 10-12-wk old BWF1 mice were treated with pCons (1 mg *i.v.*). After one-two week treatment, splenocytes were obtained both from naïve and pCons-treated BWF1 mice (2-3 mice in each group). RBC lysed, cells washed and stained with specific antibodies (CD8, CD62L, CD122, CCR7), and flow cytometry performed. **(A)** CD122 (PE) cell surface expression of naïve vs tolerized $CD8^+$ T cells treated with pCons; **(B)** CD62L (FITC) cell surface expression of naïve vs tolerized $CD8^+$ T cells pCons; **(C)** PD1 (PE) cell surface expression of naïve vs tolerized $CD8^+$ (pCons); **(D)** Percent expression of naïve $CD8^+CD62L^+$ vs tolerized $CD8^+CD62L^+$ T cells (pCons); **(E)** CCR7 (APC) cell surface expression of naïve $CD8^+$ vs tolerized $CD8^+$ T cells (pCons-treated group). Minimum 10,000 cells were gated, and live cells were used for data analyses. Dead cells were excluded from the analyses. Data were analyzed with FCS Express Ver. 7 (*De Novo*, Ontario, Canada). *p < 0.05. **(F)** Naïve $CD8^+$ and tolerized $CD8^+$ T cells were obtained, lysed, and Western Blot analysis performed with CCR7 and β-actin antibodies. CCR7 value normalized to those of β–actin. *p < 0.05.

and interferon receptor *IFNAR1* (29). In the present study, we showed herein that pCons treatment significantly downregulated and decreased (~2-5 fold) the expression of 6 genes: *RGS2*, *RGS16*, *RGS17*, *BAX*, *GPT2*, *GADD45β* (**Figures 3A–D**). We further confirmed the downregulation of GPT2, RGS16, GADD45β, and BAX proteins by Western blot analyses (**Figures 3E–H**). The downregulated BAX expression (37) in pCons-induced $CD8^+$ T cells may contribute to the survival of these cells *in vitro*/*vivo*. Overall, these data indicate that pCons regulates RGS, IFNs, and apoptotic genes.

pCons Treatment Reduces Apoptosis of $CD4^+$ and $CD8^+$ T Cells, and of $B220^+$ B Cells in BWF1 Lupus Mice

Since earlier studies demonstrated that apoptosis affects immune tolerance, we were interested to investigate whether pCons influences apoptosis in various immune cell subsets including T cells which play an important role in lupus. To address this, we treated BWF1 lupus mice with pCons. After 1-2 weeks, splenocytes were obtained, stained with Annexin V FITC, and then stained with fluorochrome-labeled $CD4^+$, $CD8^+$ and B cells specific monoclonal antibodies, and analyzed by FACS. We found that pCons treatment significantly decreased (~5 fold) apoptosis in $CD4^+$ T cells, $CD8^+$ T cells (8-10-fold) and $B220^+$B cells (~2.5-fold) compared to naïve or saline treated cells (**Figures 4A–K**). These data clearly demonstrate that pCons reduces apoptosis in both T and B cells.

pCons Increases $CD8^+FoxP3^+$ T Cells in Healthy Human Subjects

Having examined the immunomodulatory properties of pCons in murine cells, we investigated whether pCons induces $CD8^+FoxP3^+$ T regulatory cells in healthy human subjects. To determine this, we isolated PBMCs from healthy subjects and cultured them with negative control peptide (pNeg) and pCons peptide (10 μg/ml) for 24-48 hours. After culture, cells were washed, stained with CD4, CD8, CD25 and FoxP3 fluorochrome-labeled monoclonal antibodies and analyzed by flow cytometry. As shown in **Figure 5**, pCons significantly increases the mean fluorescence intensity of cells expressing FoxP3 (**Figure 5A**) and the percentage of $CD8^+FoxP3^+$ T cells are significantly increased (~5 fold) (**Figure 5B**). These data suggest that pCons induces $CD8^+FoxP3^+$ T_{reg} cells and has translational significance in humans.

DISCUSSION

Regulatory T cells play a key role in the regulation of immune responses and maintaining immune homeostasis. Impairment in the development and function of regulatory T cells is a major contributing factor in the development of autoimmune diseases, including SLE (17, 19, 38–40). Thus, inducing and regulating the function of T_{reg} is currently one of the prime goals not only in the study of autoimmune diseases, but graft versus host disease, organ transplantation, and neoplastic disease (41–43). In this study, we provided novel information for the immunological, cellular, and molecular phenotypes of regulatory T cells especially $CD8^+$ T_{reg} cells induced by the pCons treatment in BWF1 lupus mice. pCons-induced $CD8^+$ T cells express high levels of CD62L, and low levels of CD122, PD1, CTLA4, and CCR7. We did not find major changes in the expression of 41BB. Further, pCons modulated the expression of CD25, CD28, CD122, and FoxP3 in both $CD4^+$ and $CD8^+$ T_{reg} cells. The molecular phenotypes of suppressive $CD8^+$ T_{reg} cells include low level of regulator of G protein signaling *(RGS2, RGS16, RGS17), BAX, GPT2, GADD45β, PDE3b*, and *PD1* (programmed cell death 1). The downregulation of these genes in pCons-tolerized $CD8^+$ T cells was confirmed with Western blot analyses (**Figure 3**). Thus, our data revealed a molecular signature phenotype in $CD8^+$ T cells induced by pCons peptide in BWF1 lupus mice that has clinical and functional importance in the immune tolerance and their immunoregulation. For example, L-selectin (CD62L) is a type-I transmembrane glycoprotein and adhesion molecule that plays an important role in T cell activation. A recent study revealed that CD62L expression on blood basophils may predict future response to standard induction therapy for patients with lupus nephritis (44). In addition, another study found that the expression levels of CD62L decreased on T cells during the inflammatory state and levels of $CD8^+CD62L^+$ T cells negatively correlated with disease severity (45). Previously glucocorticoids have been shown to increase the CD62L expression in patients with lupus (46). In agreement with our study, a recent study also showed an increase in expression of CD62L on $CD8^+$ regulatory T cells in lupus mice (47). Thus, altogether, our finding of increased CD62L expression levels on $CD8^+$ T cells after induction by pCons treatment in BWF1 lupus mice points to a therapeutic beneficial effect.

Recent evidence suggest that both $CD8^+CD122^+$ and $CD8^+C122^-$ T cells are regulatory and can suppress autoimmunity (7, 48). Importantly, these cells express CD122 (IL-2Rβ), $CD62L^{high}$, $PD1^{low}$ and $CCR7^{low}$ (32, 49). Further, our data showed that pCons-induced $CD8^+$ T cells in BWF1 mice have less cytotoxic-T lymphocyte-antigen-4 (CTLA-4) expression as compared to naïve $CD8^+$ T cells. This is an important finding, since CTLA-4 (CD152) is an inhibitory cell-surface molecule that plays an important role in the promotion of anergy, immune regulation, and the prevention of autoimmunity. Abnormal function and susceptibility of CTLA-4 gene expression has been reported in SLE patients (50, 51). Further, it was demonstrated that CTLA-4 modulates regulatory and follicular helper T cells, thus controlling humoral immunity (52–54). CTLA-4 has been shown to downregulates CD80 and CD86 on antigen presenting cells (APC) (54). However, its precise mechanism of action has not been fully understood. Thus, our data supports the notion that pCons-induced $CD8^+$ T cells are regulatory in nature and possess all the cellular and immunological phenotypes to induce immune tolerance.

Recent reports suggest that CCR7 was involved in the progression of lupus and its expression was increased in SLE patients (55, 56). Additionally, CCR7-CCL19 couples interaction of T helper, and B cells, and dendritic cell migration (56);

thus CCR7 helps in immune complex deposition and autoantibody production. Our findings of reduction of cell surface expression of CCR7 in $CD8^+$ T cells is important because CCR7, a G protein-coupled receptor may also help in production of TGFβ. We have previously shown that pCons-induced $CD8^+$ T cells increase TGFβ mRNA and protein levels (3, 5, 26, 31); therefore, it is possible that *TGF*β may be released *via* exosomes in $CD8^+$ T cells in a CCR7-dependent manner. Exosomes are important in immunity (57), and we envision that they may play a role in $CD8^+$ T_{reg}-mediated suppression to establish and maintain self-tolerance. Earlier, we found that the mRNA of CCR7 was increased in pCons-tolerized $CD8^+$ T cells (29). These differences may be due to cell surface trafficking and or differences in transcription/translational or "half-life" of CCR7 after pCons treatment in our model system. It is also conceivable that upregulated genes and cell surface receptors, e.g., IFNAR, IFI202b, may facilitate the expression, packaging, and release of TGFβ. In contrast, downregulation of CCR7 signaling may halt the inflammatory signals in pathogenic effectors $CD4^+$ T cells, dendritic cells, antigen presenting cells (APCs), and B cells. Thus, CCR7 plays an important synergistic role in our model of immune tolerance. However, detailed mechanistic studies will be needed to address this issue.

Our findings that pCons-induced $CD8^+$ T cells have decreased level of RGS proteins are important because reduction of RGS2 signaling increases Ca^{2+} mobilization and ERK1/2 activation in response to GPCR stimulation (58) which may be contributory to the observed $CD8^+$ T_{reg} expansion and maintenance. RGS proteins are potent GTPase-activating proteins (GAP) for heterotrimeric G protein (G_q, G_i, and G_o family) alpha subunits acting as multifunctional inhibitors of signal transduction in many cells (59, 60), including "fine-tuning" GPCR signaling in lymphocytes (61). In particular, *RGS2*, also known as growth-inhibitory protein, plays a role in leukemogenesis (62). Thus, our finding that multiple RGS proteins are downregulated in tolerized $CD8^+$ T cells (**Figure 3**) reinforces the positive effect on GPCR signaling pathways, together with reduced PDE3b, that may enhance cAMP signaling and plays an important role in the suppression of T cell function (63). The downregulation of PDE3b has been associated with enhanced insulin secretion, suggesting that secretion of other factors could also be positively modulated. Consistent with the notion that reversal

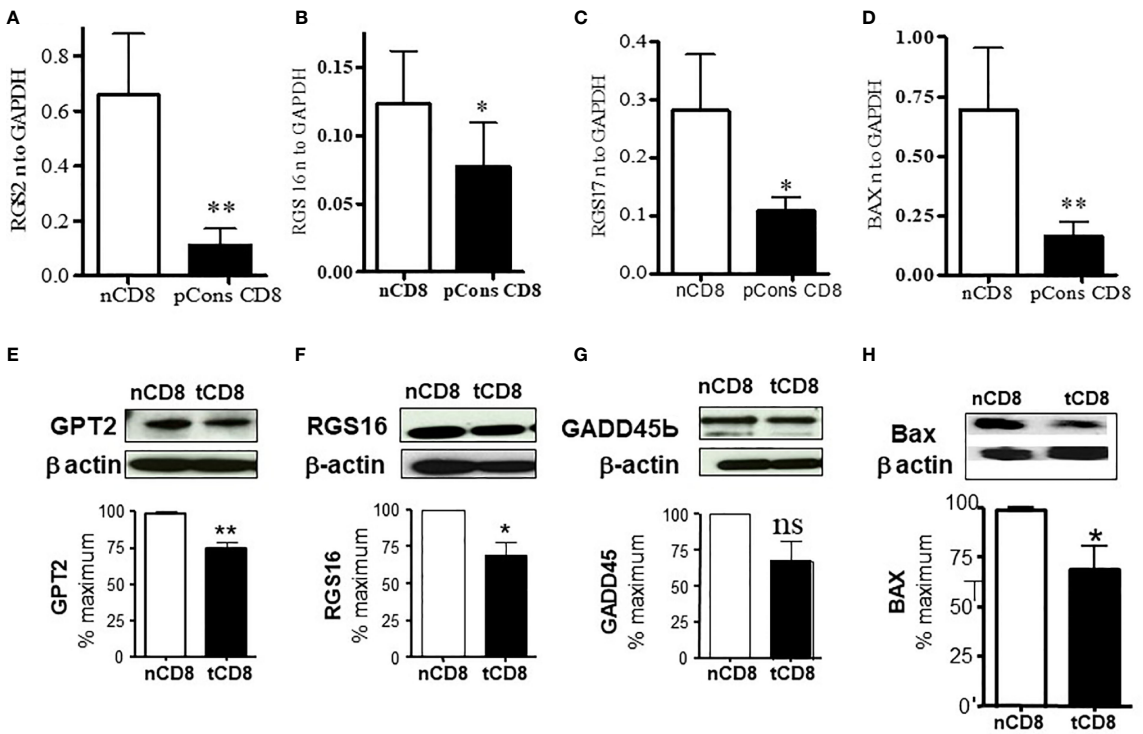


FIGURE 3 | pCons treatment downregulated the expression of Regulator of G protein signaling (RGS2, RGS16, and RGS17) and Bax (Bcl2-associated X protein) in pCons-induced $CD8^+$ T cells. Female 10-12-wk old BWF1 mice (n=3-5 mice) were treated with pCons (1 mg *i.v.*). After one-two week, splenocytes were obtained both from naïve and pCons-treated BWF1 mice. Naïve $CD8^+$ T cells and pCons-treated $CD8^+$ T cells were isolated from BWF1 mice spleen cells using microbeads from (Miltenyi Biotech (Auburn, CA, USA). Splenocytes were labelled with CD8-specific antibody, and naïve and pCons-treated $CD8^+$ T cells sorted by FACS. Cells were lysed with RNA lysis solution (Trizol) and total cellular RNA obtained. Murine primers and probes (RGS2, RGS16, RGS17, BAX, and GAPDH) were obtained from Applied Biosystems (Foster City CA, USA). Real time PCR was performed with 100 ng of RNA with gene specific primers and probes comparing naïve and pCons-treated $CD8^+$ T cells for each protein. **(A)** RGS2 normalized to GAPDH in naïve $CD8^+$ T cells vs pCons-treated $CD8^+$ T cells. **(B)** RGS16. **(C)** RGS17. **(D)** BAX. Data was normalized with GAPDH mRNA levels. **(E–H)**. Western blot analyses. **(E)** GPT2 protein level normalized to β-actin in naïve $CD8^+$ T cells vs pCons-treated $CD8^+$ T cells. **(F)** RGS16. **(G)** GADD45β. **(H)** BAX. Data was normalized to β actin protein levels. *$p < 0.05$, **$p < 0.001$. ns, not significant.

of its cAMP-degrading activity is important for maintenance of CD8$^+$ T regulatory cells. However, future studies will be required to address these possibilities.

In the current study, we found that GPT2 protein level was significantly decreased following pCons treatment in CD8$^+$ T cells in BWF1 mice. GPT2 (glutamic-pyruvic transaminase 2) also known as alanine aminotransferase (ALT) plays an important role in gluconeogenesis and amino acid homeostasis, and is an hepatic enzyme/biomarker (64) that is upregulated in disease states. GPT2 has been shown to exacerbating autoimmune disease (65), and its levels were shown to be increased in NZB/NZW F1 mice (66). Increased serum alanine aminotransferases have been reported to be associated with anti-mitochondrial antibodies in SLE patients with autoimmune liver disease (67). Further Liu *et al.* found that liver injury including increased level of ALT correlates with biomarkers of autoimmunity and disease activity in patients with SLE (68). Thus, our finding that pCons treatment reduces GPT2 protein level has both clinical and translational significance in immune tolerance of our model and in SLE.

Previously, we demonstrated that pCons-induced CD8$^+$ regulatory cells have upregulated genes including interferon inducible *202b (Ifi202b), FoxP3, Bcl2*, transformation related protein *53 (TP53)* and interferon receptor *IFNAR1* (29). We showed previously utilizing gene silencing studies that these genes are important in the suppression of anti-DNA ab in the BWF1 lupus mice (3, 5, 26, 31). In the current study, we added novel information of candidate's downregulated genes in CD8$^+$ T cells. For example, the *Bax* gene has been implicated in lupus nephritis and in apoptosis (37) suggesting that apoptosis dysregulation in SLE was affected by polymorphic variants in apoptotic-related genes including *Fas, FasL, Bcl2*, and *Bax*. While high expression of *FasL* expression contributes to increased apoptosis and to the breakdown of immune tolerance favoring autoantibody production and inflammation, low expression of the Bax protein was found to be protective in the SLE patients (69). In general, apoptotic T cells and neutrophils are increased in SLE patients and have positive correlation with SLE disease activity index (70, 71). Earlier, we showed that apoptosis was

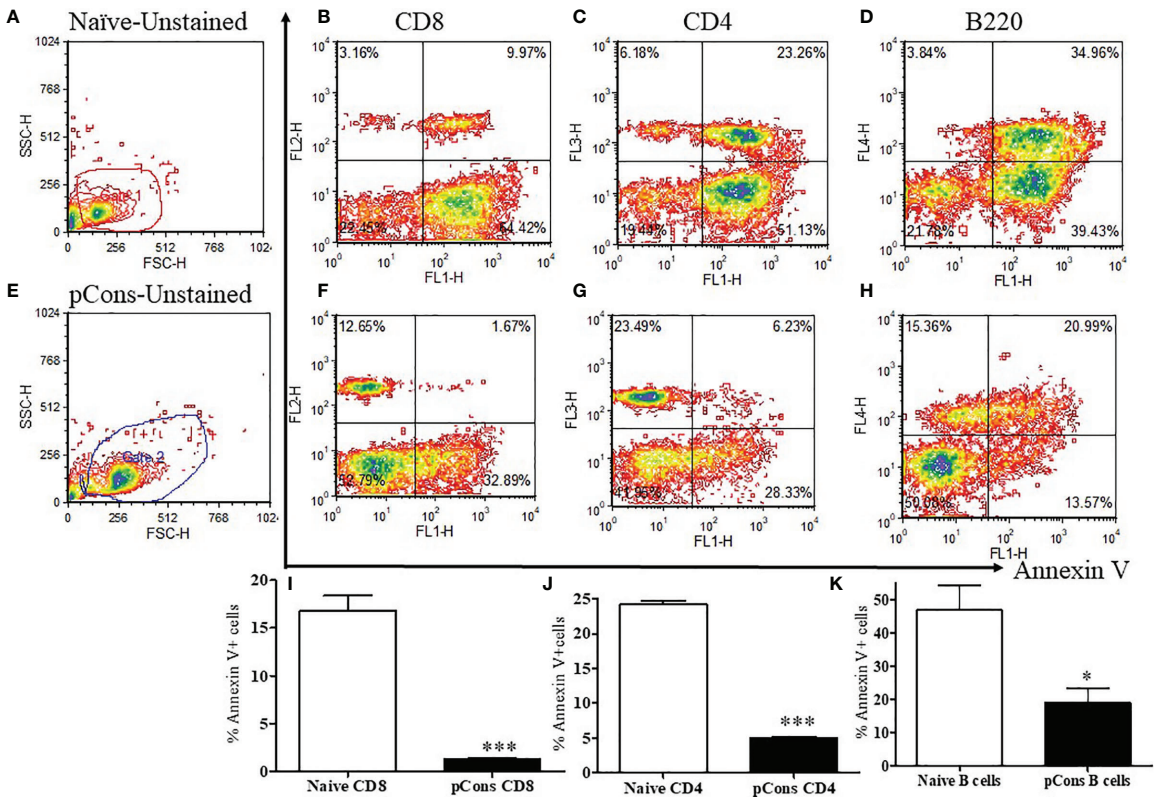


FIGURE 4 | pCons treatment alters and reduces apoptosis in both T and B cells of BWF1 mice. Female 10-12-wk old BWF1 mice were treated with pCons (1 mg *i.v.*). After one-two week treatment, splenocytes were obtained from both naïve and pCons-treated BWF1 mice (two/three mice in each group). RBC were lysed, cells washed, and stained with fluorochrome-labeled specific antibodies [CD4 (PerCP), CD8 (PE), B220 (APC), and Annexin V (FITC)] and flow cytometry performed. **(A)** Naïve unstained splenocytes; **(B)** Naïve CD8$^+$ T cells; **(C)** Naïve CD4$^+$ T cells; **(D)** Naïve B220$^+$ B cells; **(E)** pCons-treated unstained splenocytes; **(F)** pCons-treated CD8$^+$ T cells, **(G)** pCons-treated CD4$^+$ T cells; **(H)** pCons-treated B220$^+$ B cells; **(I)** Combined data of experiments (n=3-4) of naïve vs pCons-treated CD8$^+$Annexin V$^+$ T cells; **(J)** Combined data of experiments (n=3-4) of naïve vs pCons-treated CD4$^+$Annexin V$^+$ T cells; **(K)** Combined data of experiments (n=3-4) of naïve vs pCons-treated B220$^+$Annexin V$^+$ B cells. Minimum of 10,000 live cells were gated for data analyses. Dead cells were excluded from the analyses. Data was analyzed with FCS Express Ver. 7 (*De Novo*, Ontario, Canada). Statistical differences were determined by paired two-tailed Student's t-test. *$p < 0.05$, ***$p < 0.0001$.

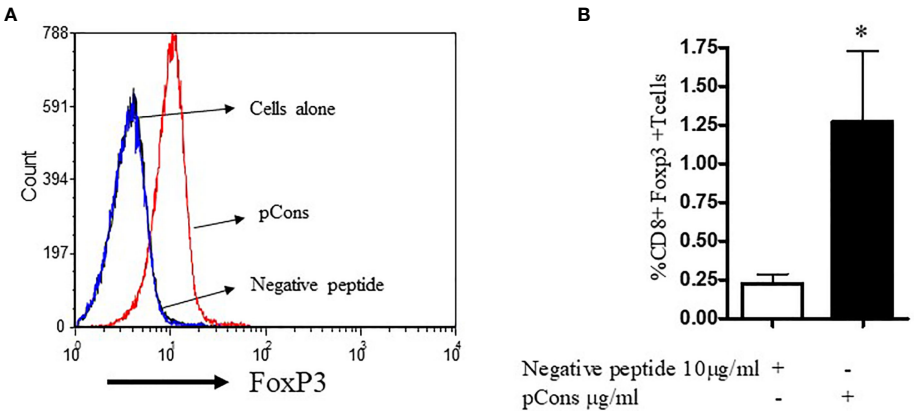


FIGURE 5 | pCons treatment increases $CD8^+FoxP3^+$ T cells in healthy subjects. $10x10^6$ peripheral blood mononuclear cells (PBMCs) were obtained from healthy volunteers (n=6). Cells were cultured with recombinant IL-2 (50 U/ml), negative control peptide A or pCons peptide (10 ug/ml) for 24-48 hours. Cells were stained with CD4, CD8, CD25 and FoxP3 fluorochrome-conjugated antibodies. FoxP3 intracellular staining was performed after cell surface staining. Cell were fixed and permeabilized as per manufacturer's protocol. Minimum 10,000 cells were gated, and only live cells were used for data analyses. Dead cells were excluded from the analyses. **(A)** Mean fluorescence intensity (MFI) of FoxP3 among naïve, negative control peptide, and pCons-treated PBMCs of healthy subjects. **(B)** Percent expression of $CD8^+FoxP3^+$ T cells between negative control peptide and pCons-treated PBMCs of healthy controls. Dead cells were excluded from the analyses. Data were analyzed with FCS Express Ver. 7 (*De Novo*, Ontario, Canada) *$p < 0.05$.

decreased in pCons-induced $CD8^+$ T cells (26). In this study, we also found decreased percent of annexin V^+ T cells and B cells in pCons-treated BWF1 mice (**Figure 4**). We postulate that pCons treatment has direct effect on both T and B cells. In addition, pCons might have an indirect effect through $CD8^+$ T_{regs}. This would require additional experiments to pinpoint the exact role. Thus, our finding of reduced Bax and annexin V in pCons-induced $CD8^+$ T cells agrees with previous studies and suggests clinical significance. Similarly, GADD45β is a critical regulator of autoimmunity (72) that plays an important role in B cell apoptosis in response to *Fas* stimulation through activation of NF-κB (73). A recent report suggests that ablation of GADD45β ameliorates the inflammation and renal fibrosis caused by unilateral ureteral obstruction (UUO) in a chronic kidney disease mouse model (74). Previously it was shown that GADD45β was also induced in $CD4^+$ T cells by inflammatory cytokines, such as IL-12 and IL-18 (75, 76). Furthermore, mRNA expression of GADD45β was associated with cytokine production and T helper cell differentiation (77, 78) and a genetic polymorphism study indicated a role for GADD45β in rheumatoid arthritis and lupus (79). Based on all these data, its plausible to hypothesize that some of the downregulated genes in pCons-induced $CD8^+$ T_{reg} cells may be regulated by specific miRNAs as these molecules have been identified with important roles in immune regulation (80). How these genes or their gene products cross-regulate in the overall suppression mechanism in our immune tolerance model has not been fully elucidated. Future detailed mechanistic studies are warranted to pinpoint the exact role.

In addition, we have shown herein that pCons peptide induces $CD8^+FoxP3^+T_{reg}$ cells in healthy human subjects suggesting translational significance. This finding is significant since patients with SLE have circulating T cells that can be activated by various peptides isolated from the variable regions of human anti-DNA antibodies (81, 82). Although, we were not able to study the modulation of $CD8^+FoxP3^+T_{regs}$ in SLE patients with pCons, it may be possible that the pCons-modulation of $CD8^+$ T_{regs} can be employed to reset the regulatory function of $CD8^+$ regulatory T cells in lupus patients. Future study will be required to address this issue.

In summary, we found that pCons treatment promoted tolerogenic immune responses and modified the various cellular and molecular phenotypes in both $CD8^+$ and $CD4^+$ T regulatory cells in BWF1 mice. The data further demonstrate that $CD8^+FoxP3^+$ T cells can be modulated by pCons peptide in human cells indicating clinical and translational significance in SLE.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the Chancellor Animal Review Committee (ARC), University of California, Los Angeles. The human study was reviewed and approved by the University of California, Los Angeles, Institutional Review Board (UCLA-IRB).

AUTHOR CONTRIBUTIONS

RPS contributed to the experimental design, obtaining funding, conducting experiments, analyzing data, preparing figures,

and writing of the manuscript. BHH contributed to funding and editing of the manuscript. DSB contributed to figure and manuscript editing. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the NIH grants AR54034, AI 083894, AI65645 to RPS; UCLA Senate Core Grant to BHH and RPS; UCLA Oppenheimer Clinical Seed Grant and American Autoimmune Related Disease Association grant to RPS.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.718359/full#supplementary-material

Supplementary Figure 1 | (A, B) Splenocytes were obtained both from naïve and pCons-treated BWF1 mice. RBC were lysed, cells washed, and stained with fluorochrome-labeled specific antibodies (CD4, CD8, CD25, CD28, CD122, CTLA-4, and 41BB). Live cell gating strategy is shown in **(A)** (panels A–D) for $CD4^+$ T cells and **(B)** (panels E-H) for $CD8^+$ T cells. Lymphocytes were first identified by a low forward scatter (FSC) and low side scatter (SSC) gate, and then further phenotyped for CD4 (CD4), CD8 (CD8a) and B (B220) cells, followed by gating for CD25, CD28, CTLA-4, 41BB, CD122. Intracellular FoxP3 expression was analyzed after cell fixation and permeabilization as per manufacturer's protocol (eBiosciences, San Diego, CA, USA).

REFERENCES

1. Filaci G, Bacilieri S, Fravega M, Monetti M, Contini P, Ghio M, et al. Impairment of CD8+ T Suppressor Cell Function in Patients With Active Systemic Lupus Erythematosus. *J Immunol* (2001) 166:6452–7. doi: 10.4049/jimmunol.166.10.6452
2. Karpouzas GA, La Cava A, Ebling FM, Singh RR, Hahn BH. Differences Between CD8+ T Cells in Lupus-Prone (NZB X NZW) F1 Mice and Healthy (BALB/c X NZW) F1 Mice may Influence Autoimmunity in the Lupus Model. *Eur J Immunol* (2004) 34:2489–99. doi: 10.1002/eji.200424978
3. Singh RP, La Cava A, Wong M, Ebling F, Hahn BH. CD8+ T Cell-Mediated Suppression of Autoimmunity in a Murine Lupus Model of Peptide-Induced Immune Tolerance Depends on Foxp3 Expression. *J Immunol* (2007) 178:7649–57. doi: 10.4049/jimmunol.178.12.7649
4. Singh RP, Hahn BH, La Cava A. Tuning Immune Suppression in Systemic Autoimmunity With Self-Derived Peptides. *Inflammation Allergy Drug Targets* (2008) 7:253–9. doi: 10.2174/187152808786848423
5. Singh RP, La Cava A, Hahn BH. Pconsensus Peptide Induces Tolerogenic CD8+ T Cells in Lupus-Prone (NZB X NZW)F1 Mice by Differentially Regulating Foxp3 and PD1 Molecules. *J Immunol* (2008) 180:2069–80. doi: 10.4049/jimmunol.180.4.2069
6. Skaggs BJ, Singh RP, Hahn BH. Induction of Immune Tolerance by Activation of CD8+ T Suppressor/Regulatory Cells in Lupus-Prone Mice. *Hum Immunol* (2008) 69:790–6. doi: 10.1016/j.humimm.2008.08.284
7. Suzuki M, Konya C, Goronzy JJ, Weyand CM. Inhibitory CD8+ T Cells in Autoimmune Disease. *Hum Immunol* (2008) 69:781–9. doi: 10.1016/j.humimm.2008.08.283
8. Kang HK, Michaels MA, Berner BR, Datta SK. Very Low-Dose Tolerance With Nucleosomal Peptides Controls Lupus and Induces Potent Regulatory T Cell Subsets. *J Immunol* (2005) 174:3247–55. doi: 10.4049/jimmunol.174.6.3247
9. Sharabi A, Azulai H, Sthoeger ZM, Mozes E. Clinical Amelioration of Murine Lupus by a Peptide Based on the Complementarity Determining Region-1 of an Autoantibody and by Cyclophosphamide: Similarities and Differences in the Mechanisms of Action. *Immunology* (2007) 121:248–57. doi: 10.1111/j.1365-2567.2007.02565.x
10. Sharabi A, Zinger H, Zborowsky M, Sthoeger ZM, Mozes E. A Peptide Based on the Complementarity-Determining Region 1 of an Autoantibody Ameliorates Lupus by Up-Regulating CD4+CD25+ Cells and TGF-Beta. *Proc Natl Acad Sci USA* (2006) 103:8810–5. doi: 10.1073/pnas.0603201103
11. Urowitz MB, Isenberg DA, Wallace DJ. Safety and Efficacy of Hcdr1 (Edratide) in Patients With Active Systemic Lupus Erythematosus: Results of Phase II Study. *Lupus Sci Med* (2015) 2:e000104. doi: 10.1136/lupus-2015-000104
12. Clemente-Casares X, Blanco J, Ambalavanan P, Yamanouchi J, Singha S, Fandos C, et al. Expanding Antigen-Specific Regulatory Networks to Treat Autoimmunity. *Nature* (2016) 530:434–40. doi: 10.1038/nature16962
13. Serra P, Santamaria P. Nanoparticle-Based Approaches to Immune Tolerance for the Treatment of Autoimmune Diseases. *Eur J Immunol* (2018) 48:751–6. doi: 10.1002/eji.201747059
14. Horwitz DA, Bickerton S, Koss M, Fahmy TM, La Cava A. Suppression of Murine Lupus by CD4+ and CD8+ Treg Cells Induced by T Cell-Targeted Nanoparticles Loaded With Interleukin-2 and Transforming Growth Factor Beta. *Arthritis Rheumatol* (2019) 71:632–40. doi: 10.1002/art.40773
15. Giang S, Horwitz DA, Bickerton S, La Cava A. Nanoparticles Engineered as Artificial Antigen-Presenting Cells Induce Human CD4(+) and CD8(+) Tregs That Are Functional in Humanized Mice. *Front Immunol* (2021) 12:628059. doi: 10.3389/fimmu.2021.628059
16. Crispin JC, Alcocer-Varela J, de Pablo P, Martinez A, Richaud-Patin Y, Alarcon-Segovia D. Immunoregulatory Defects in Patients With Systemic Lupus Erythematosus in Clinical Remission. *Lupus* (2003) 12:386–93. doi: 10.1191/0961203303lu368oa
17. Crispin JC, Martinez A, Alcocer-Varela J. Quantification of Regulatory T Cells in Patients With Systemic Lupus Erythematosus. *J Autoimmun* (2003) 21:273–6. doi: 10.1016/S0896-8411(03)00121-5
18. Liu MF, Wang CR, Fung LL, Wu CR. Decreased CD4+CD25+ T Cells in Peripheral Blood of Patients With Systemic Lupus Erythematosus. *Scand J Immunol* (2004) 59:198–202. doi: 10.1111/j.0300-9475.2004.01370.x
19. Miyara M, Amoura Z, Parizot C, Badoual C, Dorgham K, Trad S, et al. Global Natural Regulatory T Cell Depletion in Active Systemic Lupus Erythematosus. *J Immunol* (2005) 175:8392–400. doi: 10.4049/jimmunol.175.12.8392
20. Valencia X, Yarboro C, Illei G, Lipsky PE. Deficient CD4+CD25high T Regulatory Cell Function in Patients With Active Systemic Lupus Erythematosus. *J Immunol* (2007) 178:2579–88. doi: 10.4049/jimmunol.178.4.2579
21. Scalapino KJ, Tang Q, Bluestone JA, Bonyhadi ML, Daikh DI. Suppression of Disease in New Zealand Black/New Zealand White Lupus-Prone Mice by Adoptive Transfer of Ex Vivo Expanded Regulatory T Cells. *J Immunol* (2006) 177:1451–9. doi: 10.4049/jimmunol.177.3.1451
22. Horwitz DA, Gray JD, Zheng SG. The Potential of Human Regulatory T Cells Generated Ex Vivo as a Treatment for Lupus and Other Chronic Inflammatory Diseases. *Arthritis Res* (2002) 4:241–6. doi: 10.1186/ar414
23. La Cava A, Ebling FM, Hahn BH. Ig-Reactive CD4+CD25+ T Cells From Tolerized (New Zealand Black X New Zealand White)F1 Mice Suppress *In Vitro* Production of Antibodies to DNA. *J Immunol* (2004) 173:3542–8. doi: 10.4049/jimmunol.173.5.3542
24. Horwitz DA, Zheng SG, Gray JD, Wang JH, Ohtsuka K, Yamagiwa S. Regulatory T Cells Generated *Ex Vivo* as an Approach for the Therapy of Autoimmune Disease. *Semin Immunol* (2004) 16:135–43. doi: 10.1016/j.smim.2003.12.009
25. Dinesh RK, Skaggs BJ, La Cava A, Hahn BH, Singh RP. CD8+ Tregs in Lupus, Autoimmunity, and Beyond. *Autoimmun Rev* (2010) 9:560–8. doi: 10.1016/j.autrev.2010.03.006
26. Hahn BH, Singh RP, La Cava A, Ebling FM. Tolerogenic Treatment of Lupus Mice With Consensus Peptide Induces Foxp3-Expressing, Apoptosis-Resistant, TGFbeta-Secreting CD8+ T Cell Suppressors. *J Immunol* (2005) 175:7728–37. doi: 10.4049/jimmunol.175.11.7728
27. Hahn BH, Singh RR, Wong WK, Tsao BP, Bulpitt K, Ebling FM. Treatment With a Consensus Peptide Based on Amino Acid Sequences in Autoantibodies

Prevents T Cell Activation by Autoantigens and Delays Disease Onset in Murine Lupus. *Arthritis Rheum* (2001) 44:432–41. doi: 10.1002/1529-0131(200102)44:2<432::AID-ANR62>3.0.CO;2-S
28. Hahn BHA, Anderson M, La Cava A. The Anti-DNA Ig Consensus Peptide pCONS Facilitates Regulatory T Cell Activity in SLE Patients. *Arthritis Rheumatism* (2007) 56:S546–547.
29. Singh RP, Dinesh R, Elashoff D, de Vos S, Rooney RJ, Patel D, et al. Distinct Gene Signature Revealed in White Blood Cells, CD4(+) and CD8(+) T Cells in (NZBx NZW) F1 Lupus Mice After Tolerization With Anti-DNA Ig Peptide. *Genes Immun* (2010) 11:294–309. doi: 10.1038/gene.2010.6
30. Hahn BH, Anderson M, Le E, La Cava A. Anti-DNA Ig Peptides Promote Treg Cell Activity in Systemic Lupus Erythematosus Patients. *Arthritis Rheum* (2008) 58:2488–97. doi: 10.1002/art.23609
31. Dinesh R, Hahn BH, La Cava A, Singh RP. Interferon-Inducible Gene 202b Controls CD8(+) T Cell-Mediated Suppression in Anti-DNA Ig Peptide-Treated (NZB X NZW) F1 Lupus Mice. *Genes Immun* (2011) 12:360–9. doi: 10.1038/gene.2011.4
32. Liu J, Chen D, Nie GD, Dai Z. CD8(+)CD122(+) T-Cells: A Newly Emerging Regulator With Central Memory Cell Phenotypes. *Front Immunol* (2015) 6:494. doi: 10.3389/fimmu.2015.00494
33. Dai Z, Zhang S, Xie Q, Wu S, Su J, Li S, et al. Natural CD8+CD122+ T Cells are More Potent in Suppression of Allograft Rejection Than CD4+CD25+ Regulatory T Cells. *Am J Transplant* (2014) 14:39–48. doi: 10.1111/ajt.12515
34. 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
35. Suzuki M, Jagger AL, Konya C, Shimojima Y, Pryshchep S, Goronzy JJ, et al. CD8+CD45RA+CCR7+FOXP3+ T Cells With Immunosuppressive Properties: A Novel Subset of Inducible Human Regulatory T Cells. *J Immunol* (2012) 189:2118–30. doi: 10.4049/jimmunol.1200122
36. Rifa'i M, Kawamoto Y, Nakashima I, Suzuki H. Essential Roles of CD8+CD122+ Regulatory T Cells in the Maintenance of T Cell Homeostasis. *J Exp Med* (2004) 200:1123–34. doi: 10.1084/jem.20040395
37. Badillo-Almaraz I, Daza L, Avalos-Diaz E, Herrera-Esparza R. Glomerular Expression of Fas Ligand and Bax mRNA in Lupus Nephritis. *Autoimmunity* (2001) 34:283–9. doi: 10.3109/08916930109014697
38. Buckner JH. Mechanisms of Impaired Regulation by CD4(+)CD25(+)FOXP3(+) Regulatory T Cells in Human Autoimmune Diseases. *Nat Rev Immunol* (2010) 10:849–59. doi: 10.1038/nri2889
39. Mellor-Pita S, Citores MJ, Castejon R, Tutor-Ureta P, Yebra-Bango M, Andreu JL, et al. Decrease of Regulatory T Cells in Patients With Systemic Lupus Erythematosus. *Ann Rheum Dis* (2006) 65:553–4. doi: 10.1136/ard.2005.044974
40. Singh RP, Bischoff DS. Sex Hormones and Gender Influence the Expression of Markers of Regulatory T Cells in SLE Patients. *Front Immunol* (2021) 12:619268. doi: 10.3389/fimmu.2021.619268
41. Flippe L, Bezie S, Anegon I, Guillonneau C. Future Prospects for CD8(+) Regulatory T Cells in Immune Tolerance. *Immunol Rev* (2019) 292:209–24. doi: 10.1111/imr.12812
42. Tsai YG, Lee CY, Lin TY, Lin CY. CD8(+) Treg Cells Associated With Decreasing Disease Activity After Intravenous Methylprednisolone Pulse Therapy in Lupus Nephritis With Heavy Proteinuria. *PloS One* (2014) 9: e81344. doi: 10.1371/journal.pone.0081344
43. Deng Q, Luo Y, Chang C, Wu H, Ding Y, Xiao R. The Emerging Epigenetic Role of CD8+T Cells in Autoimmune Diseases: A Systematic Review. *Front Immunol* (2019) 10:856. doi: 10.3389/fimmu.2019.00856
44. Halfon M, Bachelet D, Hanouna G, Dema B, Pellefigues C, Manchon P, et al. CD62L on Blood Basophils: A First Pre-Treatment Predictor of Remission in Severe Lupus Nephritis. *Nephrol Dial Transplant* (2020) 14:gfaa263. doi: 10.1093/ndt/gfaa263
45. Liu Y, Hoang TK, Wang T, He B, Tran DQ, Zhou J, et al. Circulating L-Selectin Expressing-T Cell Subsets Correlate With the Severity of Foxp3 Deficiency Autoimmune Disease. *Int J Clin Exp Pathol* (2016) 9:899–909.
46. Tan G-z, Mao Y-p, Zeng F-q. Glucocorticoids Treatment Upregulates the Expression of CD62L in CD4+CD25+T Cells in Peripheral Blood Monocytes of Patients With Systemic Lupus Erythematosus. *Chin J Dermatol* (2007) 40:362–4.
47. Indriyanti N, Soeroso J, Khotib J. T-Cell Activation Controlling Effects of Ethyl Acetate Fraction of Kalanchoe Pinnata (Lmk) Pers on Tmpd-Treated Lupus Mice. *Int J Pharm Sci Res* (2018) 9:475–82. doi: 10.13040/IJPSR.0975-8232.9(2).475-82
48. Suzuki H, Shi Z, Okuno Y, Isobe K. Are CD8+CD122+ Cells Regulatory T Cells or Memory T Cells? *Hum Immunol* (2008) 69:751–4. doi: 10.1016/j.humimm.2008.08.285
49. Okuno Y, Murakoshi A, Negita M, Akane K, Kojima S, Suzuki H. CD8+ CD122+ Regulatory T Cells Contain Clonally Expanded Cells With Identical CDR3 Sequences of the T-Cell Receptor Beta-Chain. *Immunology* (2013) 139:309–17. doi: 10.1111/imm.12067
50. Barreto M, Santos E, Ferreira R, Fesel C, Fontes MF, Pereira C, et al. Evidence for CTLA4 as a Susceptibility Gene for Systemic Lupus Erythematosus. *Eur J Hum Genet* (2004) 12:620–6. doi: 10.1038/sj.ejhg.5201214
51. Jury EC, Flores-Borja F, Kalsi HS, Lazarus M, Isenberg DA, Mauri C, et al. Abnormal CTLA-4 Function in T Cells From Patients With Systemic Lupus Erythematosus. *Eur J Immunol* (2010) 40:569–78. doi: 10.1002/eji.200939781
52. 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
53. 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
54. 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
55. Yin S, Mao Y, Li X, Yue C, Zhou C, Huang L, et al. Hyperactivation and *in Situ* Recruitment of Inflammatory Vdelta2 T Cells Contributes to Disease Pathogenesis in Systemic Lupus Erythematosus. *Sci Rep* (2015) 5:14432. doi: 10.1038/srep14432
56. Clatworthy MR, Aronin CE, Mathews RJ, Morgan NY, Smith KG, Germain RN. Immune Complexes Stimulate CCR7-Dependent Dendritic Cell Migration to Lymph Nodes. *Nat Med* (2014) 20:1458–63. doi: 10.1038/nm.3709
57. Thery C, Zitvogel L, Amigorena S. Exosomes: Composition, Biogenesis and Function. *Nat Rev Immunol* (2002) 2:569–79. doi: 10.1038/nri855
58. Semplicini A, Lenzini L, Sartori M, Papparella I, Calo LA, Pagnin E, et al. Reduced Expression of Regulator of G-Protein Signaling 2 (RGS2) in Hypertensive Patients Increases Calcium Mobilization and ERK1/2 Phosphorylation Induced by Angiotensin II. *J Hypertens* (2006) 24:1115–24. doi: 10.1097/01.hjh.0000226202.80689.8f
59. Druey KM, Blumer KJ, Kang VH, Kehrl JH. Inhibition of G-Protein-Mediated MAP Kinase Activation by a New Mammalian Gene Family. *Nature* (1996) 379:742–6. doi: 10.1038/379742a0
60. Heximer SP, Blumer KJ. RGS Proteins: Swiss Army Knives in Seven-Transmembrane Domain Receptor Signaling Networks. *Sci STKE* (2007) 2007:pe2. doi: 10.1126/stke.3702007pe2
61. Kehrl JH. G-Protein-Coupled Receptor Signaling, RGS Proteins, and Lymphocyte Function. *Crit Rev Immunol* (2004) 24:409–23. doi: 10.1615/CritRevImmunol.v24.i6.20
62. Schwable J, Choudhary C, Thiede C, Tickenbrock L, Sargin B, Steur C, et al. RGS2 Is an Important Target Gene of Flt3-ITD Mutations in AML and Functions in Myeloid Differentiation and Leukemic Transformation. *Blood* (2005) 105:2107–14. doi: 10.1182/blood-2004-03-0940
63. Gavin MA, Rasmussen JP, Fontenot JD, Vasta V, Manganiello VC, Beavo JA, et al. Foxp3-Dependent Programme of Regulatory T-Cell Differentiation. *Nature* (2007) 445:771–5. doi: 10.1038/nature05543
64. Yang RZ, Blaileanu G, Hansen BC, Shuldiner AR, Gong DW. cDNA Cloning, Genomic Structure, Chromosomal Mapping, and Functional Expression of a Novel Human Alanine Aminotransferase. *Genomics* (2002) 79:445–50. doi: 10.1006/geno.2002.6722
65. Sundrud MS, Koralov SB, Feuerer M, Calado DP, Kozhaya AE, Rhule-Smith A, et al. Halofuginone Inhibits TH17 Cell Differentiation by Activating the Amino Acid Starvation Response. *Science* (2009) 324:1334–8. doi: 10.1126/science.1172638

66. Hsu TC, Huang CY, Chiang SY, Lai WX, Tsai CH, Tzang BS. Transglutaminase Inhibitor Cystamine Alleviates the Abnormality in Liver From NZB/W F1 Mice. *Eur J Pharmacol* (2008) 579:382–9. doi: 10.1016/j.ejphar.2007.10.059
67. Li CH, Xu PS, Wang CY, Zhang Y, Zou GL. The Presence of Anti-Mitochondrial Antibodies in Chinese Patients With Liver Involvement in Systemic Lupus Erythematosus. *Rheumatol Int* (2006) 26:697–703. doi: 10.1007/s00296-005-0034-y
68. Liu Y, Yu J, Oaks Z, Marchena-Mendez I, Francis L, Bonilla E, et al. Liver Injury Correlates With Biomarkers of Autoimmunity and Disease Activity and Represents an Organ System Involvement in Patients With Systemic Lupus Erythematosus. *Clin Immunol* (2015) 160:319–27. doi: 10.1016/j.clim.2015.07.001
69. Glesse N, Vianna P, Paim LMG, Matte MCC, Aguiar AKK, Palhano PL, et al. Evaluation of Polymorphic Variants in Apoptotic Genes and Their Role in Susceptibility and Clinical Progression to Systemic Lupus Erythematosus. *Lupus* (2017) 26:746–55. doi: 10.1177/0961203316678671
70. Yang X, Sun B, Wang H, Yin C, Wang X, Ji X. Increased Serum IL-10 in Lupus Patients Promotes Apoptosis of T Cell Subsets *via* the Caspase 8 Pathway Initiated by Fas Signaling. *J BioMed Res* (2015) 29:232–40. doi: 10.7555/JBR.29.20130037
71. Courtney PA, Crockard AD, Williamson K, Irvine AE, Kennedy RJ, Bell AL. Increased Apoptotic Peripheral Blood Neutrophils in Systemic Lupus Erythematosus: Relations With Disease Activity, Antibodies to Double Stranded DNA, and Neutropenia. *Ann Rheum Dis* (1999) 58:309–14. doi: 10.1136/ard.58.5.309
72. Liu L, Tran E, Zhao Y, Huang Y, Flavell R, Lu B. Gadd45 Beta and Gadd45 Gamma Are Critical for Regulating Autoimmunity. *J Exp Med* (2005) 202:1341–7. doi: 10.1084/jem.20051359
73. Zazzeroni F, Papa S, Algeciras-Schimnich A, Alvarez K, Melis T, Bubici C, et al. Gadd45 Beta Mediates the Protective Effects of CD40 Costimulation Against Fas-Induced Apoptosis. *Blood* (2003) 102:3270–9. doi: 10.1182/blood-2003-03-0689
74. Moon SJ, Kim JH, Choi YK, Lee CH, Hwang JH. Ablation of Gadd45beta Ameliorates the Inflammation and Renal Fibrosis Caused by Unilateral Ureteral Obstruction. *J Cell Mol Med* (2020) 24:8814–25. doi: 10.1111/jcmm.15519
75. Lu B, Ferrandino AF, Flavell RA. Gadd45beta Is Important for Perpetuating Cognate and Inflammatory Signals in T Cells. *Nat Immunol* (2004) 5:38–44. doi: 10.1038/ni1020
76. Yang J, Zhu H, Murphy TL, Ouyang W, Murphy KM. IL-18-Stimulated GADD45 Beta Required in Cytokine-Induced, But Not TCR-Induced, IFN-Gamma Production. *Nat Immunol* (2001) 2:157–64. doi: 10.1038/84264
77. Luo Y, Boyle DL, Hammaker D, Edgar M, Franzoso G, Firestein GS. Suppression of Collagen-Induced Arthritis in Growth Arrest and DNA Damage-Inducible Protein 45beta-Deficient Mice. *Arthritis Rheum* (2011) 63:2949–55. doi: 10.1002/art.30497
78. Du F, Wang L, Zhang Y, Jiang W, Sheng H, Cao Q, et al. Role of GADD45 Beta in the Regulation of Synovial Fluid T Cell Apoptosis in Rheumatoid Arthritis. *Clin Immunol* (2008) 128:238–47. doi: 10.1016/j.clim.2008.03.523
79. Li RN, Lin YZ, Pan YC, Lin CH, Tseng CC, Sung WY, et al. GADD45a and GADD45b Genes in Rheumatoid Arthritis and Systemic Lupus Erythematosus Patients. *J Clin Med* (2019) 8(6):801. doi: 10.3390/jcm8060801
80. Vinuesa CG, Rigby RJ, Yu D. Logic and Extent of miRNA-Mediated Control of Autoimmune Gene Expression. *Int Rev Immunol* (2009) 28:112–38. doi: 10.1080/08830180902934909
81. Kalsi JK, Ravirajan CT, Wiloch-Winska H, Blanco F, Longhurst CM, Williams W, et al. Analysis of Three New Idiotypes on Human Monoclonal Autoantibodies. *Lupus* (1995) 4:375–89. doi: 10.1177/096120339500400508
82. Williams WM, Staines NA, Muller S, Isenberg DA. Human T Cell Responses to Autoantibody Variable Region Peptides. *Lupus* (1995) 4:464–71. doi: 10.1177/096120339500400608

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.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

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: frontiersin.org/about/contact

REPRODUCIBILITY OF RESEARCH
Support open data and methods to enhance research reproducibility

DIGITAL PUBLISHING
Articles designed for optimal readership across devices

FOLLOW US
@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