CD4+ T CELL DIFFERENTIATION IN INFECTION: AMENDMENTS TO THE TH1/TH2 AXIOM

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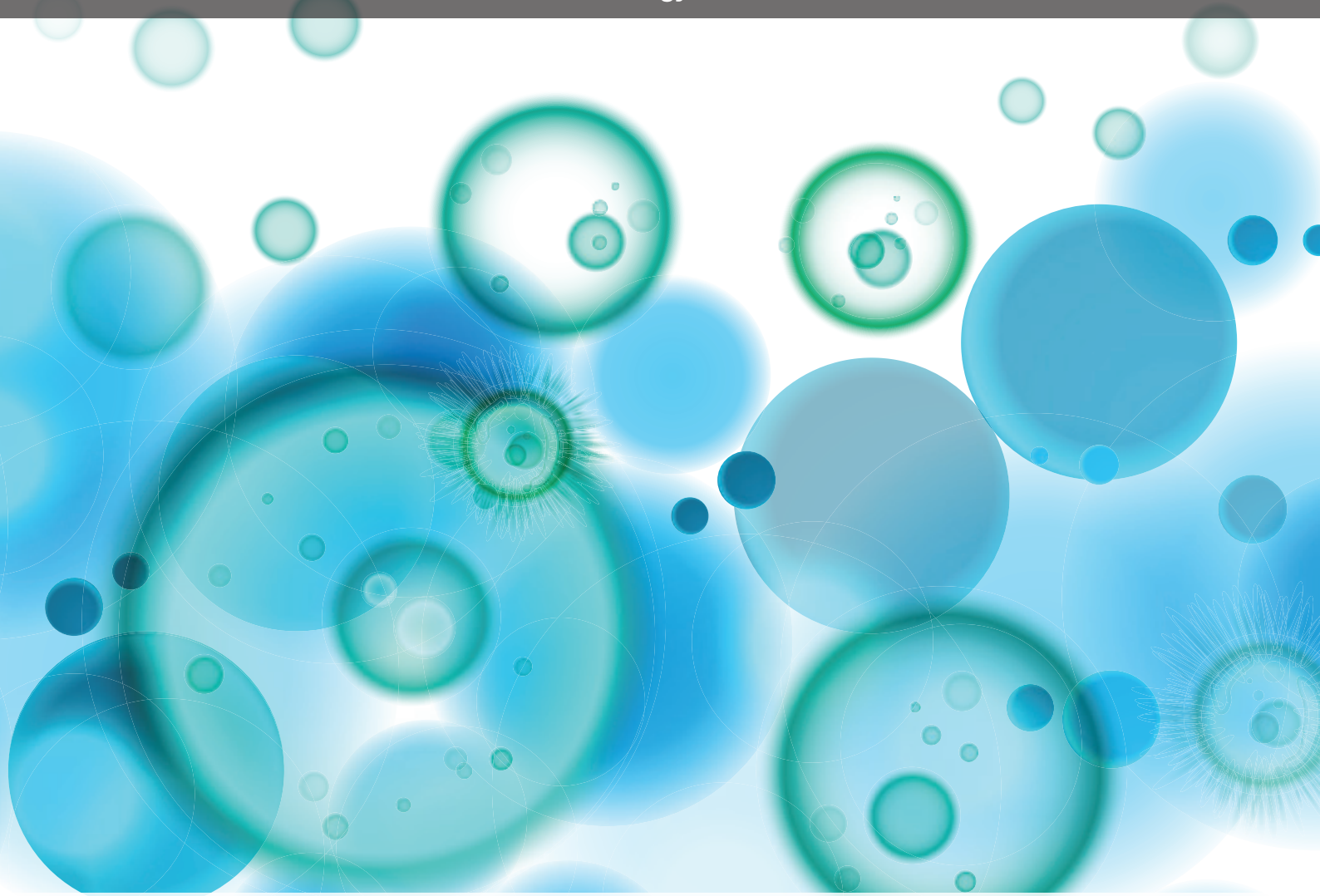

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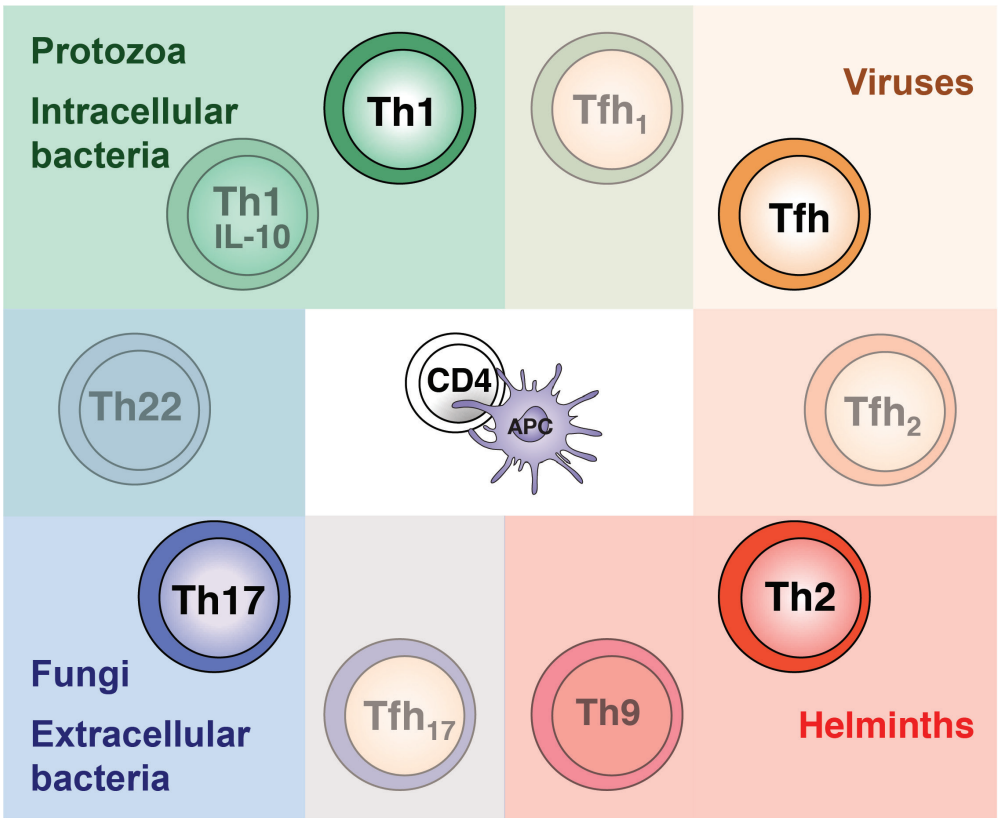
$CD4^+$ T CELL DIFFERENTIATION IN INFECTION: AMENDMENTS TO THE TH1/TH2 AXIOM

Topic Editors:
Dragana Jankovic, National Institute of Allergy and Infectious Diseases (NIAID), National Institutes of Health (NIH), Bethesda, USA
Carl G. Feng, Infectious Diseases and Immunology, Sydney Medical School, The University of Sydney, Sydney, Australia

$CD4^+$ T lymphocytes play an essential role in host defense against bacterial, parasitic and viral infections. During infection, under the influence of intrinsic signals received through peptide-MHC/TCR interactions and extrinsic signals provided by pathogen-conditioned dendritic and other accessory cells, $CD4^+$ T cells proliferate and differentiate into specialized T helper (Th) effectors, which produce distinct sets of cytokines tailored to combat a specific class of microbes.

The concept of $CD4^+$ T cell multi-functionality was developed after the seminal discovery of Th1 and Th2 cells nearly 30 years ago. Although the Th1/Th2 paradigm has successfully withstood the test of time, in the past decade additional Th subsets (Th17, Tfh, Th22, Th9) have been identified. Similarly, single cell analyses of cytokines and master transcriptional factors have revealed that, at the population level, $CD4^+$ T cell responses are far more heterogeneous than initially anticipated. While some of the checkpoints in Th cell specification have been identified, recent studies of transcriptional and epigenetic regulation have uncovered a significant flexibility during the course $CD4^+$ T lymphocyte polarization. In addition, Th cells expressing cytokines with counteracting functions, as a measure of self-regulation, display yet another level of diversity. Understanding the mechanisms that control the balance between stability vs. plasticity of Th effectors both at the time of initiation of immune response and during development of CD4 T cell memory is critical for the rational design of better vaccines and new immunotherapeutic strategies.

This research topic will cover current views on Th cell development, with a focus on the mechanisms that govern differentiation, function and regulation of effector Th cells in the context of microbial infections.



The diagram illustrates the functional heterogeneity of Th1, Th2, Th17 and Tfh cells and indicates the type of infections (color coded) with which they are associated. In addition to prototype Th effectors (non-shaded circles), Th cells with mixed effector phenotype (shaded circles) are depicted.

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CD4$^+$ T cell differentiation in infection: amendments to the Th1/Th2 axiom

Dragana Jankovic[1]* *and Carl G. Feng*[2]

[1] *Laboratory of Parasitic Diseases, National Institute of Allergy and Infectious Diseases (NIAID), National Institutes of Health (NIH), Bethesda, MD, USA,* [2] *Department of Infectious Diseases and Immunology, Sydney Medical School, The University of Sydney, Sydney, NSW, Australia*

Keywords: Th lymphocytes, cytokines, infection, dendritic cells, macrophages, immunoregulation, metabolism, memory, lnc-RNA

CD4$^+$ T helper (Th) lymphocytes play a central role in orchestrating immune responses. While the specificity of naïve CD4$^+$ T cells is fixed and constrained by the TCR they express, their effector potential is flexible and unbiased. After antigen encounter, Th lymphocytes acquire a specific effector function by responding to the summation of input signals provided by antigen-presenting cells (APC) and cytokine microenvironment. The functional diversity of Th cells provides the immune system with the capacity to mount an appropriate defense mechanism against various types of pathogens.

The initial discovery of the existence of specialized Th effector populations came from an analysis of mouse CD4$^+$ T cell clones by Mosmann and Coffman (1). This seminal study demonstrated that differentiated CD4$^+$ T cells can be classified into two groups, designated Th1 and Th2 cells, based on their cytokine production. Th1 lymphocytes, which are defined by secretion of IFN-γ, TNF, and IL-2, promote cell-mediated immunity and control infections with intracellular pathogens. In contrast, Th2 lymphocytes, which produce IL-4, IL-5, IL-10, and IL-13, mediate humoral immune responses and resistance to helminth parasites. In addition, the Th1/Th2 dichotomy was also demonstrated in immunopathological settings where Th1 and Th2 cells are implicated in autoimmune diseases and allergic conditions, respectively.

The recognition that different CD4$^+$ T cell subsets are associated with specific outcomes in both infection diseases and immune disorders propelled research into the Th1/2 paradigm. Generation of mature Th effectors was defined as an endpoint of a multistep lineage-specific differentiation process in which naïve CD4 T lymphocytes gain the ability to produce exclusively Th1 or Th2 cytokines. Moreover, a similar concept of dichotomous Th1/2 immune functions has been put forward for other lymphocyte populations (Tc1/Tc2), as well as other types of immune cells such as macrophages (M1/M2) and dendritic cells (DC1/DC2).

However, significant technical advances in CD4$^+$ T cell biology research over past 20 years have revealed that the Th1/Th2 paradigm cannot fully explain the complexity of Th effectors and led to the discovery of new Th subsets that have distinct yet overlapping functions with Th1/Th2 cells (2–4). For example, Th17 cells, which produce IL-17, are important in controlling extracellular bacterial and fungal pathogens, but can also promote autoimmune disorders (5, 6). Similarly, Tfh cells, which produce IL-21, are important for germinal center formation and antibody production, have taken on some of the functions originally attributed to Th2 cells (7, 8). Together, these findings clearly challenge Th1/2 concept and the model of Th effector choice as a bidirectional and linear differentiation process. Indeed, new molecular techniques that enable comparative analysis between genome-wide landscape of different transcriptional factors and cell-specific transcriptional output

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Edited by:
Kendall Arthur Smith, Weill Medical College of Cornell University, USA

Correspondence:
Dragana Jankovic
djankovic@niaid.nih.gov

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revealed that Th polarization is a flexible course of progressions that allows different degrees of functional specialization and diversity among Th cells.

The Research Topic presented here, "CD4 T cell differentiation in infection: an amendment to Th1/Th2 axiom" is a collection of reviews that cover the most recent progress on Th effector choice mechanisms in various infection models.

The Topic opens with two reviews on the functional dichotomy of innate immune cells. The review by Muraille et al. (9) focuses on classically (M1) and alternatively (M2) activated macrophages and their distinct metabolic programs that can be exploited by pathogens as immune evasion strategies. The paper by Hussaarts et al. (10) describes the mechanisms by which helminth components condition dendritic cells for Th2 differentiation and discusses Th2-associated inflammatory responses in the context of metabolic disorders. The immune response to intestinal worm infections is also the topic of the review by Bouchery et al. (11), which focuses on Th2-polarizing signals in $CD4^+$ T lymphocytes and the relative contribution of Th2 vs. recently discovered new Th subsets to helminth immunity. The $CD4^+$ T cells responses during fungal and malaria infection originally characterized based on Th1/Th2 paradigm are re-examined by Borghi et al. (12) and Perez-Maliah and Langhorne (13), respectively. Borghi et al. (12) describe different Th effectors implicated in anti-fungal resistance and tolerance, while Perez-Maliah and Langhorne (13) discuss the types of Th subsets, including the population of self-controlling multifunctional IL-10^+ Th1 cells, induced during malaria infection. The review by Engwerda et al. (14) focuses on the mechanisms underlying IL-10 expression in $Foxp3^-$ IL-10^+ Th1 cells and $Foxp3^+$ $CD4^+$ T regulatory lymphocytes, as well as the role these two populations play in host-protection during protozoan infection.

The notion of $CD4^+$ T cell heterogeneity beyond Th1/Th2 effectors is also supported by studies in humans as reviewed by Geginat et al. (15). The molecular mechanisms controlling plasticity vs. stability are just beginning to emerge in both murine and human Th lymphocytes. The paper by Panzeri et al. (16) presents evidence that long intergenic non-coding RNAs (lncRNA) play a role in the Th1 differentiation program of human $CD4^+$ T lymphocytes.

Compartmentalized microenvironments with tissue specific conditions (e.g., APC, cytokines) may also contribute to the plasticity of Th effectors. This is certainly true for Th differentiation in gut-associated lymphoid tissue, as discussed by Brucklacher-Waldert et al. (17). Ongoing discoveries of the variable but significant degrees of flexibility among lineage committed CD4 T cells cast doubts on "*bona fide*" Th effector memory responses. Although the Tfh effector subset is considered to be highly unstable, the review by Hale and Ahmed (18) upholds the concept that memory Tfh cells are generated during a viral infection.

The Th1/Th2 paradigm has revolutionized the concept of $CD4^+$ T cell differentiation. However, recent discoveries of additional T cell subsets have revealed previously unexpected complexity in the $CD4^+$ effector T cell decision-making process. Together, the papers presented here review our current understanding of Th effector choice in infection and emphasize the importance of defining molecular pathways dictating specificity vs. diversity and stability vs. plasticity in $CD4^+$ T cells. Ultimately, these new advances will have important implications for rational design of better vaccines and immunotherapies.

Acknowledgments

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Th1/Th2 paradigm extended: macrophage polarization as an unappreciated pathogen-driven escape mechanism?

Eric Muraille[1], Oberdan Leo[2] and Muriel Moser[2]*

[1] *Laboratory of Parasitology, Faculty of Medicine, Université Libre de Bruxelles, Brussels, Belgium*
[2] *Laboratory of Immunobiology, Faculty of Sciences, Université Libre de Bruxelles, Gosselies, Belgium*

Edited by:
Dragana Jankovic, National Institutes of Health, USA

Reviewed by:
Christopher E. Rudd, University of Cambridge, UK
António Gil Castro, University of Minho, Portugal
Hans Acha-Orbea, Center of Immunity and Infection Lausanne (CIIL), Switzerland
Thomas E. Morrison, University of Colorado School of Medicine, USA

***Correspondence:**
Muriel Moser, Université Libre de Bruxelles, Laboratory of Immunobiology, Rue des Pr Jeener et Brachet, 12, Gosselies 6041, Belgium
e-mail: mmoser@ulb.ac.be

The classical view of the Th1/Th2 paradigm posits that the pathogen nature, infectious cycle, and persistence represent key parameters controlling the choice of effector mechanisms operating during an immune response. Thus, efficient Th1 responses are triggered by replicating intracellular pathogens, while Th2 responses would control helminth infection and promote tissue repair during the resolution phase of an infectious event. However, this vision does not account for a growing body of data describing how pathogens exploit the polarization of the host immune response to their own benefit. Recently, the study of macrophages has illustrated a novel aspect of this arm race between pathogens and the immune system, and the central role of macrophages in homeostasis, repair and defense of all tissues is now fully appreciated. Like T lymphocytes, macrophages differentiate into distinct effectors including classically (M1) and alternatively (M2) activated macrophages. Interestingly, in addition to represent immune effectors, M1/M2 cells have been shown to represent potential reservoir cells to a wide range of intracellular pathogens. Subversion of macrophage cell metabolism by microbes appears as a recently uncovered immune escape strategy. Upon infection, several microbial agents have been shown to activate host metabolic pathways leading to the production of nutrients necessary to their long-term persistence in host. The purpose of this review is to summarize and discuss the strategies employed by pathogens to manipulate macrophage differentiation, and in particular their basic cell metabolism, to favor their own growth while avoiding immune control.

Keywords: macrophage polarization, metabolic switch, amino acid metabolism, hypoxia, iron, PPARs, infection, immune escape strategy

TH1/TH2 PARADIGM AND INFECTIOUS IMMUNOLOGY

Historically , immunology emerged as an independent scientific discipline whose aim was to understand and possibly ameliorate the development of vaccines, a medical practice that was mostly pioneered, in an empirical fashion, by microbiologists of the end of the 19th century. Notably, however, the establishment of fundamental immunological concepts and paradigms, such as B and T cell collaboration or tolerance, was based on a reductionist (or analytical) approach that departed from a classical "host-pathogen" view of immunology, by putting much emphasis on self/non-self discrimination concepts explored with simpler protein antigens such as ovalbumin or keyhole limpet hemocyanin (KLH).

The discovery that $CD4^+$ T cells can be characterized by distinct "cytokine signatures" by Mosmann and Coffman in 1986 (1) marked the great return of infection in immunology, leading to emergence of the Th1/Th2 "division of labor" paradigm in 1989, and its application to the field of vaccinology since the early 1990s. This concept led to a critical appraisal of the multiple and diverse sets of immune effectors promoting the now well established idea that the best immune response is often not the most intense nor the most specific but rather the best adapted to counteract or control the pathogen infectious cycle in the host. In consequence, identification of the nature of pathogens, selection of the appropriate immune response, repression of damaging or inadequate immune response are now recognized as crucial steps for the successful control of an infectious event. Thus, the immune reaction must be adapted to eliminate or control the pathogen and to restore homeostatic conditions during an infection to prevent severe tissue damage (2); for review see Ref. (3). The high frequency of chronic infections observed in nature demonstrates that pathogens are, however, able to manipulate these responses to evade immune control or even subvert immune reactions to their own advantage.

This review highlights current understanding of the interplay between pathogens and macrophages with a special emphasis on the role of metabolism regulation in the control of infections in mouse models unless otherwise stated. A clear understanding of the importance of macrophage polarization may reveal novel strategies for controlling infectious diseases.

CLASSICALLY AND ALTERNATIVELY ACTIVATED MACROPHAGE

Myelomonocytic cells are an essential component of innate immunity and represent the first line of defense against pathogens. Myelomonocytic cells fulfill a variety of homeostatic functions, which go beyond host defense and include tissue remodeling during embryogenesis, wound healing (fibrin dissolution, removal of dead tissues, fibroblast recruitment and growth and

connective-tissue remodeling), as well as orchestration and contribution to metabolic activity [reviewed in Ref. (4–6)]. Myelomonocytic cell function needs to be tailored to their tissue of residence, an adaptation that is driven by tissue-derived factors and by the physiological environment.

The myelomonocytic cell populations are particularly dynamic during inflammation or infection. Under such conditions, blood monocytes are recruited into the tissues, where they differentiate into macrophages or dendritic cells. Depending on the microenvironment, macrophages can acquire distinct functional phenotypes. The concept of macrophage polarization was first defined in 1992 with the discovery that IL-4 inhibits the respiratory burst of macrophages while enhancing expression of MHC-II and mannose receptors (CD206) on their cell surface (7). Since then, two opposite and competing phenotypes, often referred to as classically activated macrophages (M1 macrophages) and alternatively activated macrophages (M2 macrophages) [reviewed in Ref. (8–10)] have been defined and identified in several physiological settings. The M1/M2 nomenclature is derived from the Th1 and Th2 cytokines that are associated with these macrophage phenotypes.

M1 macrophages differentiate under the influence of IFN-γ and/or LPS and display functional subdivisions depending on stimulation (11). They are usually characterized by increased microbicidal activity, as witnessed by the secretion of high levels of pro-inflammatory cytokines such as TNFα and IL-6, production of reactive oxygen intermediates (ROI) and nitric oxide synthase-2 (NOS-2/iNOS)-dependent reactive nitrogen intermediates (RNI); high antigen-presenting activity and increased production of IL-12. These characteristics are promoted by IFN-γ-mediated Janus kinase–signal transducer and activator of transcription (JAK–STAT) signaling or directly by pathogen associated molecular patterns (PAMPs) such as LPS. M1 macrophages constitute the first line of defense against intracellular pathogens and promote or amplify Th1 polarization of $CD4^+$ lymphocytes by IL-12 production.

M2 macrophages have been initially identified in the context of infection by helminths, differentiating from monocytic precursors under the influence of IL-4 and IL-13 produced during a strongly Th2-polarized response. M2-like cells have been described in different pathological conditions such as infections by intracellular bacteria or virus, allergy, diabetes, and cancer [reviewed in Ref. (8, 9, 12)]. They are characterized by the selective expression of markers such as arginase 1 (Arg1), chitinase-like protein (for example, Ym1), Fizz1 (Found in Inflammatory Zone 1), CD36 (fatty acid translocase), and CD206, as well as the production of low levels of IL-12 and iNOS. Depending on the context, differences in the expression of M2-associated phenotypic markers have been recognized, leading to a redefinition of this cell population to accommodate a diverse set of subtypes: M2a (induced by exposure to IL-4 or IL-13) and M2b [induced by stimulation with immune complexes, TLR, or the IL-1 receptor antagonist (IL-1ra)] macrophages drive Th2 responses, whereas M2c cells (generated by stimulation with IL-10) play a predominant role in the suppression of immune responses and tissue remodeling [reviewed in Ref. (13)].

In addition, M1 and M2 macrophages differentially express a panel of co-stimulatory receptors of the B7 family. M1 macrophages have been shown to express higher levels of CD86 and PD-L1, whereas M2 macrophages display elevated levels of PD-L2. The expression differences are less clear for CD80 (14, 15).

It should be noted that macrophages with intermediate or overlapping phenotypes have been observed *in vivo*. For example, adipose tissue macrophages from obese mice have a mixed profile, with upregulation of several M1 and M2 gene transcripts (16). Taken together, these observations suggest that the prototypical M1 and M2 phenotypes probably represent extremes of a continuum spectrum of functionally distinct cell types. In this review, we will focus mainly on the role of M1 and M2a macrophages, these latter identified on the basis of high Arg1 expression and STAT6 pathways dependence.

MACROPHAGE POLARIZATION IS ASSOCIATED TO A SHIFT OF METABOLIC PROGRAM

M1 and M2 macrophages display a drastic shift in the amino acid, glucose, lipid, and iron metabolism [reviewed in Ref. (6, 17)]. We discuss several aspects of metabolic shift in relation to pathogen growth control.

AMINO ACID METABOLIC SHIFT

Amino acid catabolism represents a key mechanism whereby M1 and M2-types macrophages exert their anti-microbial and immunoregulatory roles. Tryptophan and arginine degradation by innate immune cells represents the most studied examples of functional links between amino acid metabolism and immune function. This relationship is quite complex, because selected degradation of amino acids can not only affect an immune response through the depletion of important precursors to protein synthesis, but also generates new catabolites endowed with immunoregulatory functions. In keeping with the general purpose of this review, we will mostly discuss herein the known links between expression and function of amino acid catabolizing enzymes and M1/M2 differentiation.

Among the three different enzymes, indoleamine 2,3-dioxygenase 1 (IDO1), 2 (IDO2), and tryptophan 2,3-dioxygenase (TDO), which catalyze oxidative Trp catabolism, IDO1 represents the best known and widely studied example illustrating how amino acid metabolism and immune regulation interface. IDO1 has been shown to confer potent anti-microbial activities *in vitro*. Through the depletion of the essential amino acid tryptophan, IDO1 can restrict *in vitro* the growth of a wide range of pathogens, including viruses, bacteria, and protozoa (18–22). Although IDO1 is constitutively expressed by numerous tissues including the epididymis, uterus, spleen, small intestines, and prostate (23), its expression is highly inducible by both type I and type II interferons, with IFN-γ considered as one of the strongest inducers of IDO expression and activity. As a consequence, and despite the fact that IDO1 expression is not restricted to hematopoietic cells, some authors have come to consider IDO1 as a prototypical M1 marker (11).

The role of IDO1 as potent anti-microbial agent *in vivo* remains, however, questionable, due in particular to the well-known immunosuppressive function of IDO1-expressing cells. Increased tryptophan catabolism has been indeed observed in the tumor microenvironment and in the placenta, contributing to tolerance to, respectively, tumor and fetal antigens. Although

apparently at odds with the anti-microbial properties of IDO1, recent observations seem to concur with the notion that despite its association to an M1-like state, IDO1 may indeed primarily mediate anti-inflammatory/regulatory roles *in vivo* opposing pathogen clearance. Indeed, genetic IDO1 ablation or pharmacological inhibition of IDO reduced parasitic load in *Leishmania major* infected mice (24). Similarly, *in vivo* administration of a pharmacological inhibitor of IDO to *Toxoplasma gondii* infected mice or infection of an IDO1-deficient mouse strain led to a more efficient control of parasite growth by IDO1-impaired hosts (25). In keeping with this prevalent immunoregulatory role of IDO1 *in vivo*, inhibition of IDO1 activity enhanced both CD4 and CD8 T cell responses to influenza virus infection, globally improving the antigen-specific memory response to this virus (26, 27).

Recent observations, albeit performed exclusively *in vitro*, have shed a new light on the possible role of IDO1 in the M1 vs M2 differentiation pathways (28). In this study, the authors demonstrate that forced expression of IDO1 in the human acute monocyte leukemia cell line THP-1 is sufficient to induce an M2-like profile, characterized by high IL-10 and low IL-12 expression pattern. Similarly, siRNA-mediated inhibition of IDO1 expression led to an increased expression of M1 markers, suggesting an important and cell autonomous role for IDO1 in regulating macrophage polarization. Collectively, and although the molecular and cellular mechanisms need to be identified, the available evidence suggests the existence of negative feedback regulatory loop whereby expression of IDO1 under M1-polarizing conditions contributes to the attenuation of a T cell mediated inflammatory response while favoring a M1 to M2 shift in macrophages.

Elevated arginine catabolism has also been linked to immunoregulation and anti-microbial immunity. Noteworthy, two of the prototypic M1 and M2 markers (respectively, iNOS and Arg1) have the capacity to use L-arginine as a substrate, leading to the production of L-citrulline and nitric oxide (NO), or L-ornithine and urea, respectively. The finding that concentrations of L-arginine at site of inflammation often decline to undetectable levels suggests an important role for L-arginine catabolism during an immune response (29, 30).

Products of Arg1 vs iNOS appear to fulfill diametrically opposed functions (**Figure 1**). Arg1 enhances collagen synthesis and cell growth via L-ornithine production, while iNOS opposes cell viability and proliferation. Moreover, iNOS protein translation appears as particularly sensitive to L-arginine levels, providing a further mechanism, in addition to competition for the same substrate, of counter-regulation between these two enzymes (31). This substrate competition between iNOS and Arg1 has been extensively described in mouse models (32), and further studies may be required to validate the universality of this concept in other species. Arginase activity has been found in lesions of patients with cutaneous leishmaniasis and in human tuberculous granulomas, suggesting an evolutionary conserved response linking arginine metabolism to infection (33–35).

M1-derived NO represents a major effector molecule in macrophage-mediated cytotoxicity, playing an important role in controlling bacterial and parasitic infections (36–38). In keeping with the previously discussed antagonistic role of Arg1 and iNOS, induction of arginase activity represents an efficient

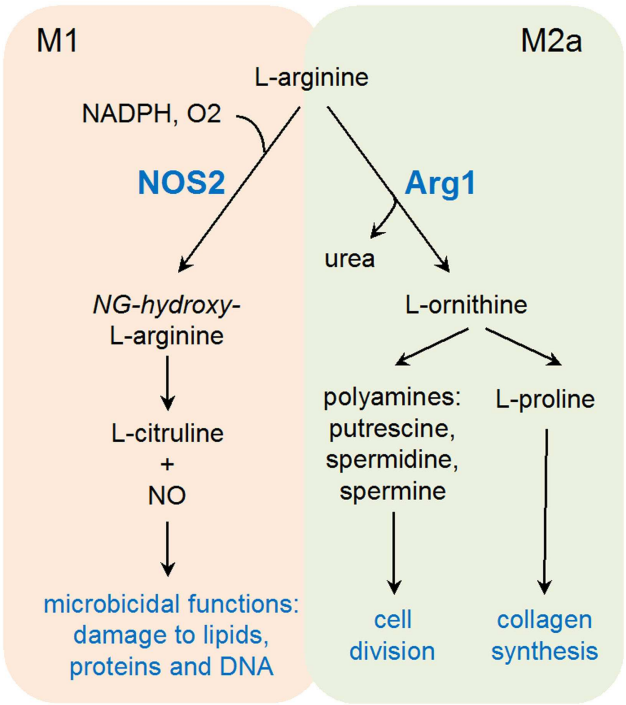

FIGURE 1 | Schematic representation of l-arginine catabolism in M1 and M2a-type macrophages.

immune escape strategy developed by several pathogens. As an example, active induction of Arg1 gene transcription in infected macrophages through manipulation of the STAT6 signaling pathway has been demonstrated for both *T. gondii* and *Leishmania donovanii* (39–41). The discovery that T cells are particularly sensitive to local arginine concentrations has further confirmed the important immunoregulatory role of Arg1, able therefore to inhibit both innate (NO production) and adaptive immune responses to parasites (30, 32). In addition to its immunoregulatory role, increased arginine metabolism has been shown to directly promote intracellular growth of several *Leishmania* species, notably through the accumulation of polyamines (42, 43). A compelling argument in favor of a role of arginine catabolism in promoting parasite growth is the expression of endogenous arginase by several parasites including *Leishmania*, *Crithidia*, and *Leptomonas* [see Ref. (44) for review].

Most of the available evidence links therefore Arg1 expression, and thus the M2 phenotype, to a disease-promoting activity. Noteworthy, however, expression of Arg1 in macrophages also plays an important role in protecting the host against the lethal effects of chronic Th2 pathology, as recently demonstrated in mice infected by *Schistosoma mansoni* (45). A similar role for Arg1 in suppressing pathologic inflammation has been derived from studies conducted in acutely *S. mansoni*-infected Arg1-deficient bone marrow chimeric mice (46). Finally, the importance of Arg1 in wound healing has been recently demonstrated using both pharmacological inhibition of arginase activity and genetic deletion of Arg1 in both haematopoietic and endothelial cells, further illustrating the

important and positive role of this enzyme in tissue repair and homeostasis (47).

Although expression of Arg1 is often associated to an immunoregulatory/pathogen-growth promoting setting, recent studies have uncovered a previously unsuspected effector role for this enzyme against nematode infection. In models of secondary infections, arginase activity was found to play a key role in the acquired protective immunity against reinfection with an intestinal helminth. Upon primary exposure to *Nippostrongylus brasiliensis*, mice developed the capacity to retain larvae in the infected skin, thus reducing the worm burden in the lungs (48). In this model, Arg1 was shown to play a key role in larvae trapping, confirming previously suspected antiparasitic effects of arginase activity against *Heligmosomoides polygyrus* (49). The capacity of the arginine catabolite L-ornithine to inhibit *H. polygyrus* larvae mobility suggests a plausible mechanism whereby Arg1 may protect the host during parasitic infections (50).

In summary, catabolism of both tryptophan and L-arginine lays at the heart of the M1/M2 dichotomy. Arginine catabolism represents an important immune mechanism limiting pathogen growth and spread, exploited by both M1 (through iNOS) and M2 (via Arg1) – like macrophages. Through distinct and mutually exclusive enzymatic reactions, the same amino acid can therefore be exploited to generate effector molecules tailored to different pathogens. Moreover, both iNOS and Arg1 have also been shown to dampen excessive inflammatory and T-cell mediated reactions. Whether these counter-regulatory properties have been evolutionary selected to control unwanted immune activity or represent unintended side effects remains to be established. In marked contrast, it is difficult at present to evaluate the precise role of IDO1 as an anti-microbial effector. Despite a large body of *in vitro* data, a role for IDO1 in limiting pathogen growth *in vivo* is still lacking, suggesting that despite being associated to an M1-like subpopulation of cells, tryptophan catabolism may primarily serve a regulatory role *in vivo*. In conclusion, amino acid catabolism elegantly illustrates the dual function of M1 and M2 macrophages, both subsets being able to express anti-microbial and immunoregulatory functions.

HYPOXIA

Decrease in oxygen pressure (hypoxia) in tissues can result from various causes such as mechanical or infection induced inflammation, intense metabolic activity but also obesity and tumor growth [reviewed in Ref. (51)]. The transcription factor hypoxia-inducible factor-1 (HIF-1) is a central mediator of hypoxic adaptation. In normoxia, HIF-1 is repressed primarily through the action of a family of hydroxylases, which targets HIF-1 subunits for degradation in an oxygen-dependent manner. In hypoxia, HIF-1 is rapidly stabilized in cells and induces the expression of hundreds of genes, which regulate angiogenesis, metabolism, growth and survival (50).

In 1938, Kempner was the first to associate cellular metabolism and inflammation: he examined the chemical composition of control or inflamed tissues and reported chemical changes that he interpreted as mainly due to the aerobic glycolysis of blood cells (52). There is indeed growing evidence that tissue foci of inflammation display a declining oxygen gradient, as compared to oxygen-rich blood stream, leading to increased cellular HIF-1α levels particularly in phagocytes, and activation of genes required to maintain viability and activity in these demanding conditions (low oxygen and glucose). It is noteworthy that HIF-1α may also exert regulatory effects in normoxia, in particular during bacterial infection, where HIF-1α expression is stimulated through TLR receptor engagement and cell signaling pathways (such as NF-κB and MAPK) [for review see Ref. (53)].

Exposure of primary human monocyte-derived macrophages to hypoxia for 16 h has been shown to result in increased mRNA levels for vascular endothelial growth factor (VEGF), glucose transporter (GLUT-1), and matrix metalloproteinase 7 (MMP-7) (54). Induction of VEGF in hypoxic conditions is involved in the pro-angiogenic activities of macrophages in tumors, whereas GLUT-1, a glucose transporter, may favor macrophage survival in ischemic tissues by increasing glucose uptake for glycolytic production of energy (**Figure 2**). The increase in mRNA coding for MMP-7 could have several consequences, as MMP-7, the smallest MMP, is able to digest many components of the basement membrane and extracellular matrix, and is involved in proteolytic processing, leading to activation of TNF-α, defensins, and other MMPs.

A number of reports have shown that hypoxia profoundly affects macrophage recruitment and development of M1 associated anti-microbial functions. Specific deletion of HIF-1α in the myeloid lineage resulted in lower ATP levels caused by decreased glycolysis. This metabolic defect results in profound impairment of myeloid cell aggregation, motility, invasiveness, and bacterial killing (55). Analysis of intracellular killing of Group B streptococci by bone marrow-derived macrophages revealed approximately sevenfold more viable bacteria within the HIF-1α deficient macrophages as compared with the wild-type cells. *In vivo*, loss of HIF-1α in myeloid cells impaired

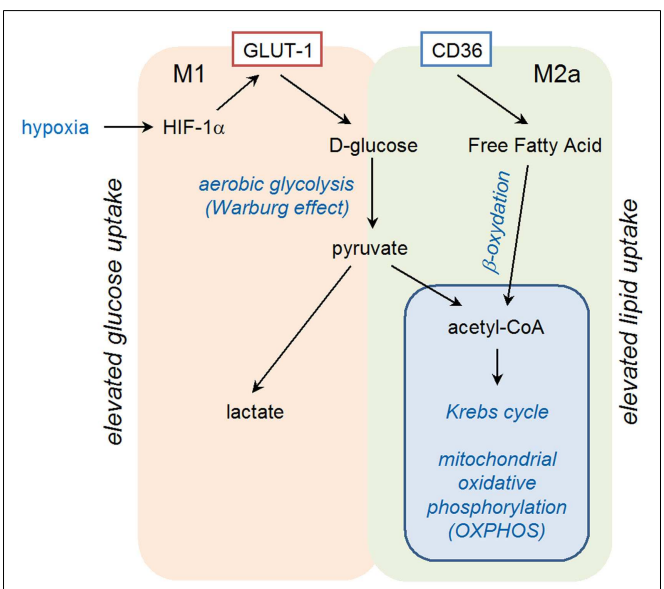

FIGURE 2 | Schematic representation of polarized metabolism in M1 and M2a-type macrophages.

infiltration at the dermal-epidermal border of inflamed skin and prevented resolution of passively induced arthritis. HIF-1α agonist mimosine and the pharmacological agent AKB-4924 (which stabilizes HIF-1) were both shown to enhance the antibacterial activity of phagocytes and to kill the pathogen *Staphylococcus aureus in vitro* and *in vivo* (56, 57). Conversely, mice lacking HIF-1α in their myeloid lineage developed larger necrotic skin lesions upon subcutaneous infection with group A *Streptococcus* (58). Murine macrophages exposed to low oxygen tensions have been shown to display M1 phenotype as suggested by increased antigen-presenting and phagocytic functions that involved IFN-γ production (59).

Collectively, these observations indicate that HIF-1α critically regulates pathways essential for the maintenance of energy homeostasis and function of macrophages in sites deprived of oxygen. However, a recent report contradicts this conclusion, as the authors found that hypoxia in *L. major* skin lesions impaired the NO-dependent leishmanicidal activity of macrophages in a reversible manner. The mechanism was found to involve impaired translation of NOS protein (60). This latter study is in accordance with two recent reports showing (i) that tumor-infiltrating macrophages in hypoxic areas displayed an altered pro-tumoral phenotype, characterized by impaired M1-type function (61) and (ii) that tumor-derived lactic acid induced *vegf* and *Arg1* and the M2-like polarization of tumor-associated macrophages in a HIF-1α-dependent manner (62).

IRON METABOLISM

Iron is an ideal redox catalyst, accepting or donating electrons, implicated in numerous cellular processes such as respiration, DNA replication but also host defenses by the production of reactive oxygen and nitrogen intermediates. Almost all living organisms from archaea to eukaryotes display absolute requirement for iron [reviewed in Ref. (63–65)]. Erythropoiesis is the most avid consumer of iron in the mammal organism as erythrocytes contain approximately 1 billion of ferric atoms. Approximately 60–70% of the human adult body iron is bound within hemoglobin (2.5 g). Macrophages play a central role in regulating iron metabolism since they recycle heme iron from senescent erythrocytes and regulate its storage. On the other hand, microbes have evoked multiple strategies to utilize iron because a sufficient supply of this metal is linked to pathogen proliferation, virulence and persistence. The expression of iron uptake systems is linked to virulence in a broad range of pathogens including bacteria, protozoa and fungi. The control over iron homeostasis is thus of central importance in host–pathogen interaction, in which both opponents compete for iron. A complex network of host proteins renders this valuable nutrient largely inaccessible to pathogens, a concept usually known as "nutritional immunity." Of course, control of intra- and extracellular pathogens requires distinct mechanisms of iron restriction in different compartments. M1 macrophages, by repressing ferroportin (a cellular iron exporter) and CD163 (a hemoglobin scavenger receptor) and inducing ferritin (which favors iron intracellular sequestration and storage), reduce the labile iron pool (LIP), the metabolically active fraction of cytosolic iron that is available for metabolic purposes. In contrast, through the upregulation of ferroportin and the downregulation of ferritin, M2 macrophages have reduced iron storage and enhanced release of iron (66). Sequestration of iron by M1 macrophages would have a bacteriostatic effect (since iron is essential to support bacterial growth) and thus represents a host protective response. Conversely, iron release from M2 macrophages would favor tissue repair. Interestingly, iron is also an important regulator of immune effector functions, immune cell proliferation and cytokine production. Iron antagonizes the IFN-γ-induced expression of MHC-II, iNOS, and TNF-α and shifts the Th1/Th2 differentiation toward a Th2 reaction.

LIPID OXIDATIVE METABOLISM

Peroxisome proliferator activated receptors (PPARs) are lipid-activated transcription factors of the nuclear hormone receptor superfamily. Endogenous ligands that regulate the PPAR family remain poorly characterized and include free native and modified fatty acids. PPARs regulate expression of target genes as obligate heterodimers with the retinoic X receptors (RXRs). In mammals, there are three PPAR isoforms (PPARα, -γ, -δ) expressed in a large variety of cells and playing pleiotropic functions in fatty acid metabolism including transport, synthesis storage, mobilization, and oxidation [reviewed in Ref. (67)].

Binding of IL-4 or IL-13 to their cognate receptors, IL-4Rα/IL-2Rγ and IL-4Rα/IL-13Rα1, respectively, initiates a cytoplasmic signaling cascade leading to tyrosine phosphorylation of transcription factor STAT6. Phosphorylated STAT6 dimerizes and translocates to the nucleus, where it induces the expression of numerous target genes. Among them, PPARγ, PPARδ, and PPARγ-coactivator-1β (PGC-1β) appear particularly important. PPARγ mediates the expression of the main M2 phenotype markers such as Arg1 and CD36 (favoring the uptake of fatty acid and apoptotic cells) and increases oxidation metabolism of fatty acid [reviewed in Ref. (68)] (**Figure 2**). By its transrepressive action, PPARγ blocks the expression of numerous pro-inflammatory mediators induced by LPS and IFN-γ such as IL-1β and iNOS. Although PPARγ is not essential for monocyte/macrophage differentiation, it functions as an important modulator of macrophage lipid metabolism and a fine-tuner of immune functions (**Figure 3**). Whereas the instructions for alternative macrophage activation are provided by IL-4 and IL-13, the acquisition and long-term maintenance of this phenotype implicate PPARγ and PPARδ. PPARγ KO mice are deficient in M2 macrophages, develop spontaneous chronic Th1 inflammation in lung (69) and are more susceptible to obesity and insulin resistance, suggesting that homeostatic functions performed by M2 macrophages might allow animals to more efficiently store and oxidize incoming lipids, thereby maintaining insulin sensitivity and glucose tolerance by attenuating inflammation (70).

Reciprocally, fatty acids facilitate the acquisition and maintenance of M2 phenotype (**Figure 3**). In a PPARδ-dependent manner, monounsaturated fatty acids, such as oleic acid, were found to synergize with IL-4 to enhance the expression of alternative activation signature genes such as Arg1 in macrophages. Arg1 promoter is directly activated by PPAR/RXR heterodimers [reviewed in Ref. (67)] providing a molecular explanation for the observed synergy between nutrients and Il-4/IL-13 signaling pathways.

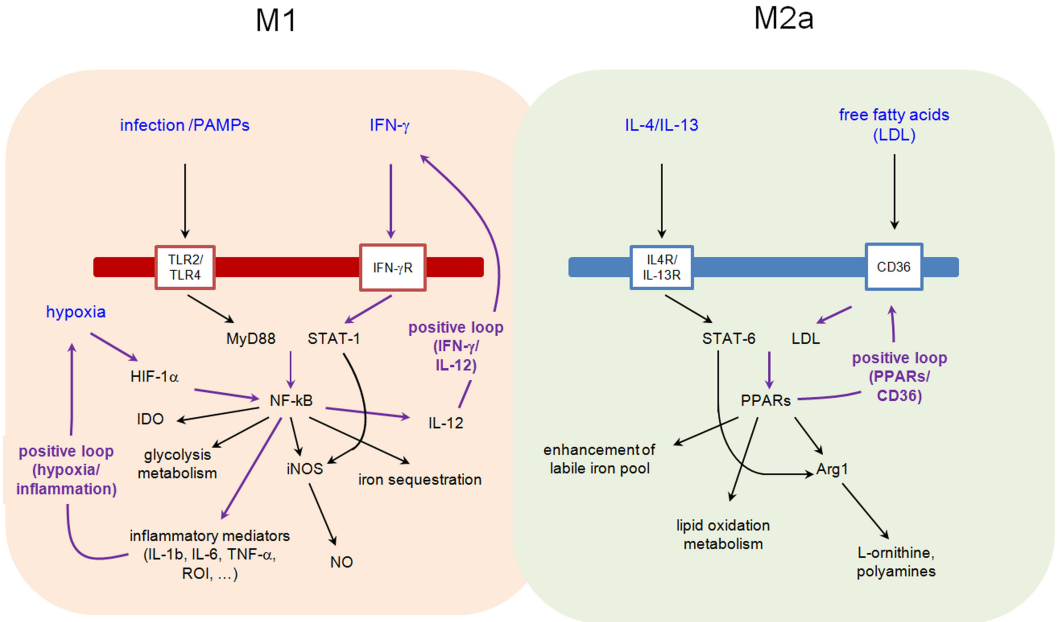

FIGURE 3 | Interplay between metabolic pathways and M1 and M2a functions.

THE ENERGY SENSORS: AMPK AND mTOR

The AMP-activated kinase (AMPK) represents a well described energy sensor able to adapt cell metabolism to available substrates and/or cellular energetic requirements. Accumulation of cellular AMP, indicative of a reduction in the energy status of the cell, activates the AMPK, which in turn will phosphorylate a series of substrates to favor metabolic reactions (such as stimulation of mitochondrial biogenesis, oxidative metabolism and autophagy), while decreasing ATP consuming, biosynthetic pathways. The mammalian target of rapamycin (mTOR) plays a similar role, by adapting metabolic reactions (such as protein synthesis) to nutrient availability in order to preserve cellular homeostasis. Pathogens have been shown to manipulate these regulatory pathways to highjack cell metabolism to their own benefit [reviewed in Ref. (71, 72)]. Collectively, these studies have highlighted how, by inhibiting AMPK and/or maintaining an activated mTOR pathway, pathogens generate essential substrates for their own replication. Notably, a link between macrophage metabolism and inflammation has also been recently established. A reduction in macrophage AMPK activity is associated with M1-like, pro-inflammatory status, while increased AMPK activity promotes an anti-inflammatory, M2-type, response [for review see Ref. (73)]. Inhibition of AMPK expression or activity promotes expression of pro-inflammatory mediators in macrophages activated by LPS (74). Similarly, AMPKα1 KO macrophages failed to acquire an M2 phenotype, retaining high levels of iNOS expression even when stimulated in the presence of M2-polarizing factors such as IL-4 (75).

In summary, polarization states and functional properties of macrophages largely depend on environmental conditions, such as hypoxia, cytokines, pathogen-derived TLR-ligands, and lipid mediators. Metabolism shift associated to macrophage polarization appears highly adapted to microbicidal and tissue regeneration function of M1 and M2 macrophages, respectively.

M2-LIKE POLARIZATION AS A PATHOGEN-INDUCED IMMUNE ESCAPE STRATEGY

The interaction between pathogenic microbes and their host is determined by survival strategies to compete for essential nutrients. During evolution, microbes have developed strategies to access selected nutrients from the host, while these, in return, have devised defensive means to restrict their availability to pathogens. Long-term pathogen persistence is frequently the consequence of a subtle equilibrium between the nutritive needs of host and pathogens. M2 macrophages appear as a favorable niche for long-term persistence of numerous intracellular pathogens. Some evidences suggest that intracellular pathogens can induce or benefit from PPAR expression and/or high iron availability in infected macrophages.

Mycobacterium tuberculosis, the agent of tuberculosis, is well adapted to survive within the hostile macrophage environment. Current evidence (76) indicates that *M. tuberculosis* and BCG infection causes a time-dependent upregulation of PPARγ expression in infected macrophages. Even uptake of dead bacteria triggered PPARγ expression in macrophages, suggesting a pattern recognition receptor-mediated triggering mechanism. Accordingly, recent studies have shown the implication of TLR2 (77) and mannose receptor (CD206) (78) in this process. PPARγ expression and activation led to increased lipid droplet formation, expression of M2 markers and downmodulation of bactericidal M1 response including respiratory burst and NO production. Involvement of CD36 in lipid droplet formation was further confirmed by decreased BCG-induced lipid droplet formation in CD36 deficient macrophages (77). These observations suggest that PPARγ

expression may not only represent an escape mechanism to circumvent the protective host response, but may also provide the nutrient rich environment required for mycobacterial growth and survival by promoting host lipid metabolism.

A key anti-pathogen effector of M1 macrophages is NO, which is involved in direct killing of pathogens, such as *M. tuberculosis*. Interestingly, a report has shown that Arg1 was induced in macrophages *in vitro* via a STAT6 independent and TLR dependent pathway, and prevented NO production. The absence of Arg1 was associated with increased NO expression and enhanced control of mycobacteria. These observations suggest that chronic infection could be linked to pathogen-induced Arg1 expression (37). Conversely, abrogation of Arg1 expression in hypoxic macrophages where iNOS was rendered ineffective resulted in exacerbated lung granuloma pathology and bacterial burden, suggesting that Arg1 could also play a positive role in the control of *M. tuberculosis* (79).

Iron availability in macrophage is also crucial for *Mycobacteria* growth (64, 65). Iron overload enhanced susceptibility while iron-poor diets reduced *Mycobacteria* virulence in mice (80). The depletion of intracellular iron occurs in M1 polarized macrophages. However, *Mycobacterium* produces siderophores (mycobactins) with incredible high affinity to iron able to remove iron from transferrin and lactoferrin, leading to augmentation of iron concentrations in infected macrophages favoring *Mycobacterium* growth. As previously mentioned, intracellular iron accumulation could also downregulate M1 phenotype by negatively affecting iNOS expression (81).

Brucella spp. are facultative intracellular α-proteobacteria and the causative agent of the world's leading zoonotic disease brucellosis (82). Its pathogenesis is mainly based on its ability to survive and multiply inside macrophages. Treating *Brucella abortus*-infected mice with a PPARγ inhibitor led to a significant decrease in splenic colonization during the chronic phase of infection (83). In contrast, treatment of *B. abortus*-infected mice with PPARγ agonist led to increased expression of M2 markers in splenic macrophages and enhanced bacterial count in the spleen during the chronic infection phase (83). These results strongly suggest that pathways downstream of PPARγ contribute to generate a niche for persistence of *B. abortus*. In the same study (83), further analysis of *in vitro* infected macrophages suggested that PPARγ favors intracellular glucose availability for *Brucella* by shifting the energetic cell metabolism from a typical M1-associated glycolytic pathway to a glucose-sparing lipid oxidation associated to M2 differentiation.

Although iron is needed by bacteria for growth, it also plays an important role in the anti-microbial M1 activities of macrophages such as generation of hydroxyl radicals. *Brucella* appears well equipped to deal with the iron-poor environment in M1 macrophages. *Brucella* expresses two siderophores [2,3-dihydroxybenzoic acid (2,3-DHBA) and 2,3-DHBA-based molecule brucebactin] and a heme oxygenase (BhuO) allowing the use of heme as an iron source [reviewed in Ref. (84)]. It has been observed that iron supplementation increased the ability of macrophages to control intracellular *Brucella* (85) suggesting that iron chelation may represent a valuable strategy whereby this bacterium impedes the generation of toxic hydroxyl radicals.

Salmonella are Gram-negative bacteria infecting hosts via the gastrointestinal tract. Host-adapted strains of *Salmonella enterica* cause systemic infections (typhoid fever) and have the ability to asymptomatically persist within host tissues for long periods of time in 1–6% of patients [reviewed in Ref. (86)]. Early protective Th1 responses are followed by pathogen-permissive Th2 responses. *Salmonella* infection caused accumulation and persistence of hemophagocyte macrophages (characterized by the ingestion of non-apoptotic cells) expressing M2 markers (CD36 and CD206) in spleen (87). During later stages of infection *in vivo*, *Salmonella* was found preferentially associated with these cells (88), possibly representing a specific niche for persistent infection. PPARδ was found upregulated in *Salmonella* infected macrophages *in vitro*. PPARδ deficiency dramatically inhibited *Salmonella* replication *in vitro* and *in vivo* and its pharmacological activation enhanced bacterial growth in mice. Absence of PPARδ was associated to a decrease of available glucose necessary to *Salmonella* persistence in macrophages (88). How macrophages acquire and/or maintain an M2 phenotype *in vivo* during *Salmonella* infection is presently unknown.

Leishmania represent obligate intracellular protozoan parasites that are transmitted in the dermis of the mammalian host by blood-feeding sand flies. After transmission, these parasites invade mainly macrophages, which are decisive effector cells that either kill or host the intracellular parasites depending on the balance of iNOS and Arg1 [reviewed in Ref. (89)]. Control of parasite growth was associated with the induction of strong Th1 responses and induction of iNOS-expressing M1 macrophage. Accordingly, there was a clear direct correlation between the parasite load and the arginase activity in the lesions. Inhibition of arginase activity during the course of infection reduced parasite growth (43). *In vitro*, parasitized dendritic cells showed coordinated transcriptional modulations that correlated in part to PPARχ upregulation and promoted the generation and storage of neutral lipids, such as triacyl-sn-glycerol and cholesteryl esters (90), that can be important for the synthesis of key parasite membrane components. Accumulation of neutral lipid has been also shown to reduce antigen processing and presentation to effector T cells (91). *Leishmania* parasites are coated with phosphatidylserine (92), a major surface characteristic of apoptotic cells (93) recognized by CD36 expressed by M2 macrophages (94). As engulfment of apoptotic cells by CD36 pathways leads to induction of PPAR, it is possible that *Leishmania* actively promotes M2 polarization of macrophages as a virulence strategy. Of note, a recent report shows that the macrophage response upon visceral infection with *Leishmania infantum* is characterized by an M2b-like phenotype, identified by expression of C-type lectin receptors signature (95).

Francisella tularensis, a gram-negative intracellular bacteria and the causative agent of tularemia, induces acute and frequently lethal pulmonary infection. *F. tularensis* replicates in myeloid cells and alveolar epithelial cells in lung of infected mice [reviewed in (96)]. It has been observed that virulent strains induce M2 activation markers on macrophages in wild type but not in IL-4Rα or STAT6 deficient mice (97). Lipid isolated from virulent strain but not from attenuated strain appeared able to induce a TLR2/PPARγ-dependent M2 polarization of macrophage (98),

suggesting that redirection of macrophage polarization by *F. tularensis* could constitute an escape immune mechanism.

Listeria monocytogenes is a facultative intracellular Gram-positive bacteria able of serious acute infection. *Listeria* infection of macrophages rapidly led to increased expression of PPARγ (99). Selective loss of PPARγ in myeloid cells resulted in enhanced innate immune defense against *Listeria* both, *in vitro* and *in vivo* suggesting that PPARγ-dependent M2 phenotype could favor *L. monocytogenes* multiplication.

T. gondii represents one of clear examples of how an intracellular parasite can subvert macrophage polarization to enhance its infectivity. This parasite secretes into the cytoplasm of infected macrophage several virulence factors including a protein, ROP16, displaying protein kinase activity. Remarkably, this protein interferes with the host signaling machinery through the direct phosphorylation of STAT6 (and STAT3), bypassing therefore early cytokine receptor proximal events. Notably, ROP16 was shown to phosphorylate STAT6 on the critical activation residue Tyr641 (39), resulting in the productive transcription of M2-associated genes including Arg1 (41, 100).

In conclusion, numerous evidences suggest that some intracellular bacterial and protozoan pathogens responsible for chronic (*Mycobacteria, Brucella, Leishmania*, and *Toxoplasma*) but also acute (*Francisella* and *Listeria*) infection actively manipulate STAT6-PPARγ/δ pathways to avoid M1 polarization of macrophages and/or benefit from a nutrient rich environment associated to lipid oxidation metabolism. Although the possible impact of macrophage polarization on chronic viral infections remains largely unknown, recent studies suggest that HBV-induced M2 macrophage polarization can participate to immune impairment and pathology (101). Thus, PPARγ/δ pathways hold a pivotal role in the establishment of chronic infection and their ligands may be used as combination therapeutics to limit host pathology or pathogen persistence.

BENEFICIAL ROLE OF Th2 RESPONSE AND ALTERNATIVELY ACTIVATED MACROPHAGE DURING INFECTION

It is well appreciated that M2 macrophages can act to limit disease severity and protect the host from detrimental effects of an excessive Th1 response, making symbiotic survival between host and parasites more likely. For example, infection of mice or humans with the trematode *S. mansoni* results in a Th2 dependent response. Neutralization of IL-4 or genetic invalidation of IL-4/IL-13 led to increased rates of mortality during acute schistosomiasis, illustrating the protective role of Th2 in this experimental model. Mice rendered selectively deficient for IL-4R in macrophages were extremely susceptible to infection with 100% mortality, suggesting that M2 macrophages are essential during schistosomiasis for protection against organ injury.

In addition, M2 macrophages can also display anti-microbial effector function and actively reduce the level of infection by various pathogens.

Infective larvae of the nematode *N. brasiliensis* enter host animals through skin penetration and further migrate in the lungs and intestine. Primary protective response against *N. brasiliensis* relied mostly on an IL-13-dependent goblet cell hyperplasia leading to increased mucus production in the intestine. Accordingly, IL-4Rα and STAT6 deficient mice failed to expulse the gastrointestinal nematode parasite (102). Interestingly, protective memory response against *N. brasiliensis* implicated neutralization of larva migration from skin to lung (48). Larval trapping was dependent on induction of M2 macrophage differentiation from recruited monocytes as demonstrated by the fact that neutralization of IL-4 or Arg1 abolished larval trapping.

The central pathophysiologic events in severe malaria caused by *Plasmodium falciparum* are the inability of the host defenses to control parasite replication resulting in the excessive release of pro-inflammatory cytokines. Mice treated with an agonist of PPARγ had reduced parasitemia of *Plasmodium* and decreased inflammatory response (103). This effect was mediated by CD36 that served as ligand for *P. falciparum* erythrocyte membrane protein 1 (PfEMP-1) expressed by the parasite (104). CD36-mediated internalization by M2 macrophages facilitated removal of neutrophils and dead tissues to resolve inflammation as well as clearing of the parasite. *P. falciparum* used host hemoglobin as iron source and sequestered heme into a pigment known as hemozoin. It has been showed that hemozoin reacted with membrane phospholipids to generate hydroxy-polyunsaturated fatty acids which are ligands of PPARγ (105), suggesting that the parasite can actively modulate its growth by a PPARγ pathway.

The cestode *Mesocestoides corti* induces a central nervous system infection in mice dominated by M2 macrophages. Absence of STAT6 signaling resulted in enhanced susceptibility to infection coinciding with increased parasite burden in the brain (106) suggesting that M2 macrophages actively control infection.

The ubiquitous fungus *Aspergillus fumigatus* causes invasive and allergenic disease. Host defense relied on the ability of the respiratory immune system to restrict spore germination into invasive hyphae and to limit fungus-induced or inflammation-induced damage in infected tissues. Infection induced a M2 polarization of alveolar macrophages. Control of infection appeared dependent on dectin-1 mediated phagocytosis of fungus by M2 macrophages (107) as dectin-1 deficiency or elimination of M2 macrophages was associated to increased fungal burden in mice.

PLASTICITY OF MACROPHAGE POLARIZATION DURING INFECTION: TWO OPPOSITE EXAMPLES

Several infectious experimental models demonstrate that immune polarization may display high plasticity, suggesting that M1 and M2 phenotypes may not represent terminally differentiated cells.

Classically, M1 macrophages are implicated in initiating and sustaining inflammation, whereas M2 macrophages differentiate later and are involved in resolution of inflammation and tissue regeneration. In particular, the early and late phases of *Trypanosoma congolense* infection are characterized by M1 and M2 polarization of macrophages, respectively. The shift to M2 appears indispensable to control inflammation and limit tissue injury (105).

However, the opposite scenario has also been reported. *Cryptococcus neoformans*, the etiological agent of cryptococcosis constitutes a well-documented case of M2-to-M1 shift *in vivo*. After inhalation, *C. neoformans* resides primarily in the alveolar spaces, where it can survive and replicate in the extracellular lung

environment. The immune response in *C. neoformans*-infected C57BL/6 wild type mice is predominantly Th2 biased and associated with development of M2 macrophages. These latter serve as intracellular reservoirs for the microbe and promote the development of lung pathology (108). In this model, neutralization of IFN-γ resulted in more severe pulmonary infections and lesions (109). In contrast, IL-4 (109) or IL-13 (110) deletion led to Th1-dominated immune responses associated with M1 macrophage granuloma formation displaying iNOS dependent fungicidal activity. Interestingly, the lung immune polarization environment changed overtime (108). Following a strong initial induction of Th2 cytokines in the lungs at 2 and 3 weeks post-infection, a considerable increase in Th1 cytokines occurred at 5 weeks post-infection. This increase in Th1 cytokines was accompanied by a decrease in Th2 cytokines, indicating that the immune system is spontaneously capable of a Th2–Th1 shift. However, if it is now appreciated that polarized T cells exhibit previously unsuspected flexibility and plasticity (111), it remains unclear whether macrophage polarization switches involve recruitment of new precursors or de-differentiation of macrophages *in situ*.

Collectively, these observations suggest that macrophages can undergo dynamic transitions between different functional states. This polarization switch may provide protection to uncontrolled inflammation or constitute an indispensable adaptation to rapid phenotypic change of pathogen during its cycle.

CONCLUSIONS

In summary, the available evidences are compatible with the original view of a "division of labor" between M1 and M2 macrophages. M1 macrophages do display an increased microbicidal activity against a wide range of intracellular parasites, while differentiation toward an M2-like state is often observed during the resolution phase of an inflammatory response, favoring in particular tissue repair. This purely dualistic view needs, however, to be considered with care, since M1 macrophages are also able to negatively control an inflammatory response (through notably NO production and IDO1-mediated tryptophan catabolism), while M2 macrophages display anti-microbial activities via expression of Arg1 (**Figure 4**). The M1/M2 cell paradigm represents, therefore, an additional example of pathogen-tailored immune effectors, endowed with regulatory properties.

A growing number of clinically relevant infectious diseases are characterized by pathogen persistence in the host. Chronic and recurrent infections implicate long-lasting and costly therapy and are the cause of an important morbidity in the world. Therefore, there is a strong interest in understanding the biology of pathogen persistence.

Numerous reports demonstrate that chronic bacterial persistence *in vivo* depends on the ability of bacteria to resist the anti-microbial activity but also to reconfigure local host environment to their profit (112). Pathogens exploit host regulatory pathways limiting damaging inflammatory responses for the benefit of tolerance. They also reprogram host cell metabolism to produce nutrients necessary for their long-term persistence (88, 113). Similar results have been obtained in parasitic infections (12, 89) suggesting that induction of a metabolic shift in infected cells constitutes a general strategy to favor persistence. In this paradigm,

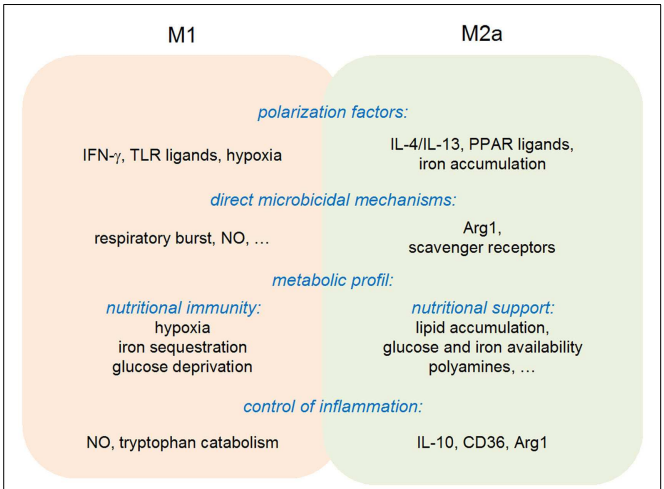

FIGURE 4 | Multipolar view of M1 and M2a polarization.

PPARs family seems key actor in the hijacking of macrophages by pathogens and an interesting target for therapeutic strategy.

Targeting microbial nutrient requirements represents a promising therapeutic strategy. Both anti-microbial compounds and vaccine have been created to specifically target pathogen iron acquisition systems [reviewed in Ref. (65)]. Unfortunately, microbes often possess redundant mechanisms for nutrient acquisition. A better understanding of these mechanisms may lead to the development of new strategies to control infection.

The importance of nutrient availability in control of infection also leads to focus our attention on the multifactorial and complex impact of modern fat rich diet on chronic infection. Commensal and mutualistic microorganisms are present in all mucosal compartments and form the microbiota. These symbiotic organisms compete with pathogenic microorganisms for nutrients, thereby preventing pathogenic colonization and invasion. As discussed above, obesity can affect macrophage polarization but also drastically change the composition of host microbial community and thus its ability to compete with pathogen for host nutriment availability. A better knowledge of the relationship between nutrition, microbiota, and immune defense could provide guidelines for prophylactic nutritional measures to protect against and/or treat infections.

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Priming dendritic cells for Th2 polarization: lessons learned from helminths and implications for metabolic disorders

Leonie Hussaarts[1], Maria Yazdanbakhsh[1] and Bruno Guigas[1,2]*

[1] *Department of Parasitology, Leiden University Medical Center, Leiden, Netherlands*
[2] *Department of Molecular Cell Biology, Leiden University Medical Center, Leiden, Netherlands*

Edited by:
Dragana Jankovic, National Institutes of Health, USA

Reviewed by:
António Gil Castro, University of Minho, Portugal
David Escors, University College London, UK

***Correspondence:**
Bruno Guigas, Department of Parasitology, Leiden University Medical Center, P.O. Box 9600, Postzone L40-Q, Leiden 2300 RC, Netherlands
e-mail: b.g.a.guigas@lumc.nl

Nearly one quarter of the world's population is infected with helminth parasites. A common feature of helminth infections is the manifestation of a type 2 immune response, characterized by T helper 2 (Th2) cells that mediate anti-helminth immunity. In addition, recent literature describes a close association between type 2 immune responses and wound repair, suggesting that a Th2 response may concurrently mediate repair of parasite-induced damage. The molecular mechanisms that govern Th2 responses are poorly understood, although it is clear that dendritic cells (DCs), which are the most efficient antigen-presenting cells in the immune system, play a central role. Here, we review the molecular mechanisms by which DCs polarize Th2 cells, examining both helminth antigens and helminth-mediated tissue damage as Th2-inducing triggers. Finally, we discuss the implication of these findings in the context of metabolic disorders, as recent literature indicates that various aspects of the Th2-associated inflammatory response contribute to metabolic homeostasis.

Keywords: antigen-presenting cells, dendritic cells, helminth, Th2 cells, obesity, metabolic diseases, insulin resistance, type 2 inflammation

INTRODUCTION

Helminths are parasitic worms that infect one quarter of the world's population. They classically evoke strong type 2 immune responses characterized by the induction of T helper 2 (Th2) cells, which secrete cytokines like IL-4, IL-5, and IL-13. These promote IgE production by B cells, and recruitment of eosinophils and alternatively activated macrophages. Together, these events control infection and/or mediate parasite expulsion through smooth muscle contraction and mucus production [reviewed in Ref. (1, 2)].

Helminths enter, migrate, and exit through their host, causing considerable tissue damage along the way. Therefore, it may not be surprising that recent literature has described a close association between type 2 immune responses and wound repair (3–6). In this context, a Th2-cell associated response would contribute to both wound repair and control of parasite infection, and seems beneficial over a type 1 response, which harbors a greater risk of inducing collateral tissue damage (1). In addition, various aspects of the type 2 immune response have been shown to contribute to metabolic homeostasis (7). Indeed, helminths were recently found to protect against diet-induced insulin resistance (8, 9), and a negative association exists between helminth infection and metabolic syndrome (10).

The mechanisms that initiate Th2 responses are not fully understood, even though it is clear that dendritic cells (DCs), the most efficient antigen-presenting cells (APCs) in the immune system, play a crucial role (11). Since helminths are the strongest natural inducers of type 2 immune responses, many advances in dissecting the mechanisms underlying Th2 polarization have been made using either models of helminth infection or helminth-derived products. In this Mini Review, we discuss recent advances in the field, examining both helminth antigens and helminth-mediated tissue damage as triggers for the initiation of a Th2 response. In addition, we discuss the potential implications of these findings in the context of metabolic disorders.

DENDRITIC CELL SUBSETS ASSOCIATED WITH Th2 POLARIZATION

The importance of DCs in Th2 skewing is highlighted by studies showing that depletion of $CD11c^+$ DCs interferes with the induction of a Th2 response to *Schistosoma mansoni* and *Heligmosomoides polygyrus* (12–14). Interestingly, it has become increasingly clear that distinct DC subsets induce different Th responses [reviewed in Ref. (11, 15)], and in the last few years, several studies analyzed the role of DC subsets in the initiation of Th2 responses to helminth infection. For example, two independent groups recently showed that the development of a Th2 response to *Nippostrongylus brasiliensis* depends on dermal $CD301b^+$ DCs (16, 17). Specifically, depletion of $CD301b^+$ DCs prior to infection reduces IL-4 production by $CD4^+$ T cells, without affecting the percentage of T follicular helper (Tfh) cells or germinal center B cells (16). Mechanistically, Th2-inducing $PDL2^+CD301b^+$ DCs were shown to depend on DC-specific expression of the transcription factor interferon regulatory factor 4 (IRF4) (17). In line with these findings, $CD11c^{int}MHCII^{hi}$ dermal DCs expressing PDL2 and CD301b were also identified as a Th2-priming DC subset in *N. brasiliensis* infection (18). Of note, $CD301b^+$ DCs alone are insufficient to generate a Th2 response *in vitro* (17) or *in vivo* (16), suggesting that additional requirements exist. For example, optimal localization of DCs within the lymph node may play a crucial role. In *H. polygyrus* infection, CXCR5-expressing $CD11c^+$ DCs migrate to the lymph node and localize adjacent

to B cell follicles (19). Depletion of CXCR5 or B cell-derived lymphotoxin alters the localization of the DCs and, as a consequence, impairs the development of Tfh and Th2 cells (19). In addition, it has been suggested that DCs require signals from basophils (20) and group 2 innate lymphoid cells (ILC2s) (21) to prime Th2 responses to allergens. Together, these studies suggest that specific DC subsets, as well as the microenvironment in which these subsets encounter $CD4^+$ T cells, are important for Th2 development *in vivo*.

PRIMING DENDRITIC CELLS FOR Th2 POLARIZATION

SENSING HELMINTH-DERIVED ANTIGENS

Dendritic cells are equipped with pattern recognition receptors (PRRs) that recognize a wide array of pathogen-associated molecular patterns (PAMPs). The classical paradigm describes that triggering of PRRs, including the Toll-like receptors (TLRs), RIG-I-like receptors, NOD-like receptors, scavenger receptors, and C-type lectin receptors (CLRs), induces DC maturation and subsequent antigen-specific activation of Th cells (22).

While signaling through most TLRs induces Th1/Th17 responses (23), Th2-inducing helminth-derived molecules have also been described to interact with DCs through TLR2, 3, and 4 (24–27). Although the schistosome-related glycan LNFPIII, which contains Lewis X (Le^X) trisaccharides, requires TLR4 for Th2 skewing (28), various studies suggest that TLRs are dispensable for Th2 polarization by helminth antigens. For example, bone marrow-derived DCs (BMDCs) from TLR2- and TLR4-knockout mice can still skew Th2 when pulsed with *S. mansoni* soluble egg antigens (SEA) (29), and the TLR adaptor protein MyD88 is not required for Th2 skewing by SEA-stimulated splenic DCs (30). Interestingly, human monocyte-derived dendritic cells (moDCs) stimulated with phosphatidylserine lipids from schistosomes induce IL-10-producing T cells through TLR2 (25). Therefore, helminth products may employ TLRs for the induction of regulatory responses, but it seems that other PRRs are required for the initiation of a Th2 response.

Indeed, CLRs that sense helminth glycans play an important role in Th2 skewing. For example, SEA is internalized by moDCs through DC-specific ICAM-3-grabbing non-integrin (DC-SIGN), macrophage galactose-type lectin (MGL), and mannose receptor (MR) (31), and binds to Dectin-2 on BMDCs (32). Binding of SEA to DC-SIGN was shown to depend on Le^X (33), and a recent study showed that blocking DC-SIGN-associated signaling inhibits Th2 skewing (34). Likewise, excretory/secretory products from the tapeworm *Taenia crassiceps* (TcES) bind MR and MGL on BMDCs (35), and the Th2-skewing capacity of TcES is glycan-dependent (36). Since SEA and ES mixtures contain many different glycoproteins, it is difficult to pinpoint the receptor and/or the mechanism responsible for Th2 polarization. Therefore, an important contribution to the field was made when omega-1, a small glycoprotein expressing Le^X residues (37), was identified as the major immunomodulatory component in SEA (38, 39). Generation of a glycosylation mutant revealed that omega-1 requires its glycans to condition moDCs for Th2 skewing, and to prime Th2 responses both *in vitro* and *in vivo*. Specifically, MR but not DC-SIGN, mediates recognition and internalization of omega-1 (40). In sum, these studies indicate that helminth-derived antigen preparations can bind a variety of PRRs, which may induce distinct intracellular events that promote Th2 polarization.

SENSING EPITHELIAL ALARMINS

Modulation of DCs for Th2 priming can also take place in the absence of PRR signals, in response to epithelium-derived cytokine alarmins that are released with tissue damage (41). For example, stimulation of human myeloid DCs with thymic stromal lymphopoietin (TSLP) primes naïve T cells to produce IL-4, IL-5, IL-13, and tumor necrosis factor alpha (TNF-α) (42). However, the role of TSLP in helminth infection remains controversial. While TSLP receptor (TSLPR) knockout mice fail to mount a protective Th2 response to *Trichuris muris* (43, 44), they do develop a Th2 response during infection with *S. mansoni* (45), *H. polygyrus* or *N. brasiliensis* (44). Interestingly, basophils rather than DCs were recently described to act as TSLP-dependent APCs for Th2 skewing in *Trichinella spiralis* infection (46).

A second relevant alarmin is IL-33, as stimulation of BMDCs with this cytokine promotes Th2 development (47, 48). In line with these findings, IL-33 treatment improves Th2 cytokine production and expulsion of *T. muris* (49), and mice deficient for the IL-33 receptor T1/ST2 fail to develop a Th2 response following injection with *S. mansoni* eggs (50). Importantly, T1/ST2 is not only present on DCs but also on lymphocyte subsets including ILC2s, which were shown to mediate *N. brasiliensis* expulsion in an IL-33-dependent manner (51).

Lastly, IL-25 induces the production of type 2 cytokines by ILCs, and IL-25-knockout mice show delayed initiation of type 2 cytokine responses and *N. brasiliensis* expulsion (52). Although IL-25 has not been described to act directly on DCs, it was shown to enhance cytokine production by Th2 memory cells activated by TSLP-conditioned myeloid DCs (53). Thus, multiple alarmins are released by epithelial cells and may act in concert on various immune cell types, to mediate the induction of a Th2 response against helminths or their eggs.

INTRACELLULAR MECHANISMS ASSOCIATED WITH Th2 POLARIZATION

Signaling-dependent mechanisms

Pattern recognition receptor-mediated signaling classically induces DC maturation via mitogen-activated protein kinases (MAPK) (54). However, in contrast to microbial ligands, helminth products often fail to induce classical signs of maturation and are well-known to downregulate TLR-mediated maturation (31, 38, 55–60). Indeed, unlike many TLR ligands, Th2-inducing compounds fail to phosphorylate p38 MAPK but instead promote phosphorylation of p42/p44 MAPK (ERK1/2) [reviewed in Ref. (61)]. ERK1/2 stabilizes c-Fos, and inhibiting either c-Fos or ERK1/2 enhances IL-12 production by moDCs (62), suggesting that activation of this pathway suppresses Th1-polarizing cytokines. Likewise, TSLP promotes ERK1/2 phosphorylation (63) and fails to induce IL-12 production by myeloid DCs (42, 64).

It was noted that the NF-κB signaling pathway also seems to be involved in Th2 polarization, as SEA- or LNFPIII-stimulated BMDCs from NF-κB1 knockout mice fail to prime a Th2 response (65, 66). Furthermore, it was recently demonstrated that Le^X residues, via DC-SIGN, activate LSP1 in moDCs, leading to nuclear

accumulation of the atypical NF-κB family member Bcl3 and downregulation of IL-12 mRNA. These events also seem required for SEA-induced T cell polarization, since silencing either LSP1 or Bcl3 interferes with Th2 skewing (34). Similarly, the Th2-inducing capacity of TSLP was shown to involve activation of NF-κB and STAT5 (63, 67).

Finally, SEA can signal through spleen tyrosine kinase (Syk) downstream of Dectin-2, activating the Nlrp3 inflammasome and increasing TLR-triggered release of IL-1β by BMDCs. However, infection of various inflammasome-deficient mice with *S. mansoni* demonstrated that activation of this pathway does not seem to favor any particular Th response (32). Thus, helminth antigens can activate signaling, and certain members of the NF-κB and ERK pathways in particular seem to play a role in Th2 polarization.

Signaling-independent mechanisms

In addition to signaling-dependent mechanisms, various helminth products harbor enzymatic activities that mediate Th2 skewing. For example, omega-1 depends on its RNase activity, which allows the molecule to cleave both ribosomal and messenger RNA, to downmodulate TLR-induced moDC maturation and IL-12 production, and to skew toward Th2 (40). Interestingly, various Th2-inducing allergens are also RNases (68, 69), as well as the endogenous eosinophil-derived neurotoxin that can amplify DC-mediated Th2 polarization (70). Together, these reports suggest that any RNase internalized by DCs may harbor Th2-priming capacities, through cleavage of ribosomal and/or messenger RNA. Similarly, a number of studies identified a role for cysteine protease inhibitors secreted by filarial nematodes (cystatins) in regulating host immune responses by interfering with antigen processing [reviewed in Ref. (71)]. Therefore, helminths may employ both signaling-dependent and independent mechanisms to condition DCs for Th2 skewing (**Figure 1**).

Of note, recent studies indicate that modulation of metabolic pathways within immune cells can regulate their function and, thereby, the outcome of the immune response (72). For example, BMDCs switch their core metabolism from mitochondrial oxidative phosphorylation to glycolysis upon TLR-ligation, and inhibition of this switch interferes with maturation, IL-12 expression, and the ability to induce $CD4^+$ T cell proliferation (73, 74). Among the underlying mechanisms, the mammalian target of rapamycin (mTOR) was shown to control glycolytic metabolism (75, 76). Although we recently showed that mTOR is not involved in Th2 skewing by omega-1- or SEA-conditioned moDCs (77), the question whether helminths or their products affect glycolytic

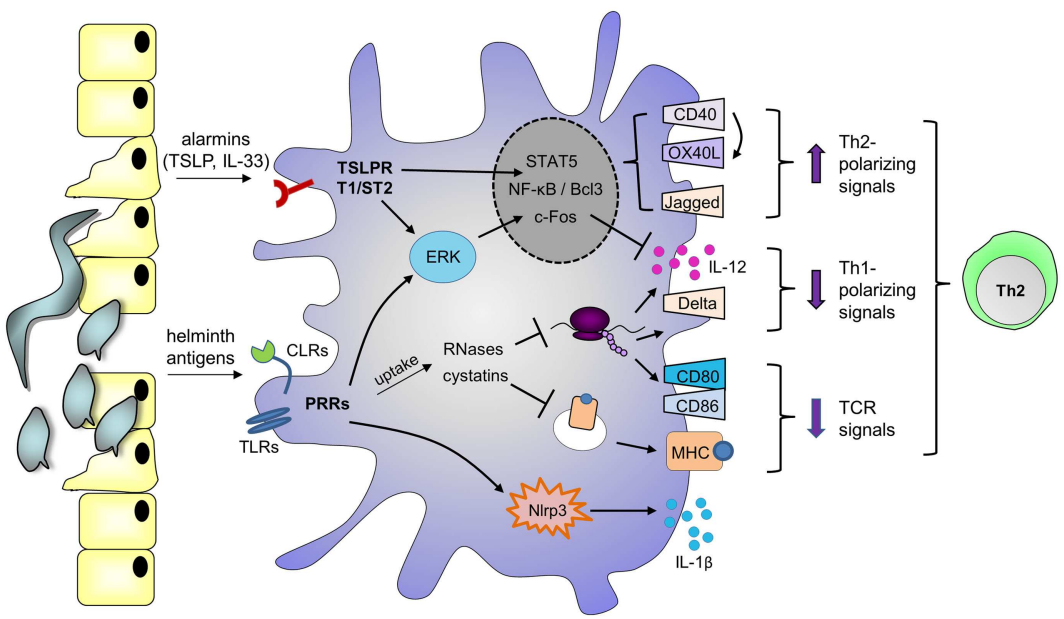

FIGURE 1 | Possible mechanisms by which helminth molecules modulate DCs for Th2 polarization. Helminth antigens are recognized by DCs through ligation of pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs) and C-type lectin receptors (CLRs). Depending on the antigen, binding promotes phosphorylation of ERK1/2, nuclear accumulation of NF-κB or Bcl3, and/or activation of the Nlrp3 inflammasome, which mediates IL-1β secretion. Phosphorylation of ERK1/2 stabilizes c-Fos, leading to downregulation of IL-12 expression. In addition, DCs can upregulate expression of Th2-associated CD40 and Jagged, which are under the control of NF-κB and ERK1/2, respectively (115, 116). Upon encounter of T cells expressing CD40L, signaling through CD40 promotes OX40L expression in an autocrine manner. Alternatively, PRRs may mediate uptake of antigens that interfere with antigen presentation on MHCs, such as cystatins, or RNases that inhibit protein synthesis, thereby suppressing the expression of costimulatory molecules like CD80 and CD86. These events affect T cell receptor (TCR) signaling. As a consequence of protein synthesis inhibition, RNases may also downregulate Th1-polarizing cytokines or molecules like IL-12 and Delta-4. In parallel, helminths or their eggs damage epithelium, and alarmins are released, such as thymic stromal lymphopoietin (TSLP) and IL-33, which bind the TSLP receptor (TSLPR) and T1/ST2, respectively. TSLP can also activate ERK1/2, STAT5, and NF-κB to promote CD40 and OX40L expression. Altogether, these events favor DC-mediated Th2 polarization.

reprograming in DCs, and how this relates to Th2 polarization, constitutes an exciting new area of research.

PRIMED DCs AND INITIATION OF T CELL POLARIZATION

A major difference between Th1 and Th2 development is that a Th1 response requires persistent production of Th1-polarizing cytokines, like IL-12, which are exclusively produced by APCs. By contrast, once primed DCs induce IL-4 production by a few activated Th cells, the Th2 response is self-sustained through autocrine production of IL-4 (78, 79). Therefore, in order to understand mechanisms of Th2 polarization, it is critical to identify the DC-associated polarizing signals that control early IL-4 production by activated T cells.

SOLUBLE FACTORS AND SURFACE MOLECULES

As discussed above, DCs stimulated with helminth molecules or TSLP fail to express IL-12. Moreover, injection of IL-12 can block the development of a Th2 response to *S. mansoni* eggs (80). These findings led to the so-called "default concept," which states that Th2 differentiation spontaneously occurs in the absence of a Th1-priming signal like IL-12. However, mice lacking IL-12 do not develop a Th2 response to microbial pathogens (81), and blocking the mTOR pathway in LPS-stimulated moDCs skews a potent Th2 response without suppressing IL-12 secretion (77), suggesting that there are active signals involved in Th2 differentiation.

Such a signal may be provided by a soluble factor secreted by DCs, like RELMα, which was shown to promote IL-10 and IL-13 secretion by lymph node cells following adoptive transfer of SEA-stimulated BMDCs (82). However, supernatants from SEA-primed moDCs do not skew toward Th2 (83), and neither SEA- nor omega-1-stimulated BMDCs induce Th2 when separated from $CD4^+$ T cells in transwells (39), indicating that an active polarizing signal in these studies is likely provided by surface molecules. Indeed, the Notch ligands Delta-4 and Jagged-2 have been linked to Th1 and Th2 polarization, respectively (84), and helminth antigens were shown to upregulate Jagged-2 on BMDCs (85, 86) and to suppress Delta-4 expression in moDCs (87). However, Jagged-2-deficient BMDCs can still skew Th2 when challenged with SEA (85, 86), suggesting that other molecules may be involved. For example, CD40 has been proposed to provide a polarizing signal, as its expression on SEA-stimulated BMDCs is required for the induction of a Th2 response (88), and mice lacking CD40 ligand suffer from impaired Th2 development during *S. mansoni* infection (89). Mechanistically, signaling through CD40 promotes OX40L expression, which is essential for optimal Th2 skewing by SEA-conditioned BMDCs (90) and moDCs (83), as well as TSLP-conditioned myeloid DCs (64). However, treatment with anti-OX40L does not significantly affect the Th2 response to *N. brasiliensis* infection (18), and it has been suggested that OX40L acts as a costimulatory molecule rather than a polarizing signal, since SEA-treated OX40L-knockout DCs induce Th2 cells, but fail to stimulate appropriate T cell expansion (90). Altogether, these studies suggest that there may not be one specific DC-associated molecule required for Th2 polarization, but rather a combination of signals that mediate both optimal T cell priming and expansion.

ROLE FOR THE T CELL RECEPTOR

Early reports have described that the antigen dose can determine the outcome of Th differentiation, with a high dose generally favoring Th1 development (91–93). These findings were confirmed in a recent report, which also indicated that Th1-inducing adjuvants promote a higher Ca^{2+} flux [representing T cell receptor (TCR)-signaling strength], and induce larger synapse size, than Th2-promoting molecules (94). In addition, it has been suggested that T cells activated by Th2-inducing ligands are less proliferative, as priming of splenic DCs with SEA reduces the frequency of $CD4^+$ T cells progressing through the cell cycle, and drug-induced arrest of cell cycle progression promotes Th2 polarization (30). Together, these observations suggest that helminth molecules may reduce TCR triggering, impairing T cell proliferation in favor of Th2 differentiation. Indeed, treatment of splenic DCs with SEA results in shorter T cell–DC interaction times and lower TCR signaling when compared to a Th1-inducing adjuvant (94). In addition, omega-1 reduces the capacity of BMDCs to form T cell–DC conjugates and diminishes the frequency of $CD4^+$ T cells progressing through the cell cycle, possibly through modification of actin morphology (39). Mechanistically, interaction between T cells and DCs was shown to depend at least in part on the costimulatory molecule CD80 (94). As discussed above, helminth products fail to induce upregulation of costimulatory molecules, which may also explain why DCs treated with helminth molecules are less capable of forming stable interactions with T cells.

IMPLICATIONS FOR METABOLIC DISORDERS

The induction of a type 2 immune response has multiple functions. In the context of helminth infection, it mediates both parasite clearance and enhances wound healing. In addition, it has long been known that type 2 inflammatory responses contribute to the pathogenesis of allergy and asthma (95). Recently, however, it has become clear that multiple facets of the type 2 immune response are also involved in metabolic regulation (7). For just one example, IL-4 can regulate the balance between fatty acid and glucose oxidation in hepatocytes (96). Studying the molecular mechanisms that helminths employ to govern Th2 polarization may therefore open novel avenues for the treatment of metabolic disorders.

METABOLIC DISORDERS AND TYPE 2 INFLAMMATION

A growing body of literature indicates that obesity is associated with chronic low-grade inflammation in metabolic organs. Enhanced infiltration of classically activated M1 macrophages, $CD8^+$ T cells, and Th1 cells has been reported in both liver and adipose tissues (AT) (97). This represents a key etiological mechanism promoting tissue-specific insulin resistance and impairment in whole-body glucose homeostasis, which leads to an increased risk for type 2 diabetes and cardiovascular diseases. Interestingly, various reports have shown that Th2-inducing conditions, such as *N. brasiliensis* infection (8, 9), allergic inflammation (96), or SEA administration (98), improve insulin sensitivity and glucose tolerance in diet-induced obese mice. In addition, both *S. mansoni* infection (99) and SEA administration (100) reduce the development of atherosclerotic lesions in mice. Furthermore, adoptive transfer of $CD4^+$ T cells (mostly via Th2 cells) and IL-4 treatment can protect against diet-induced insulin resistance (96, 101).

Lastly, type 2-associated ILC2s (102, 103) and eosinophils (8) were shown to play a crucial role in maintenance of whole-body metabolic homeostasis by sustaining AT alternatively activated M2 macrophages. These findings are in line with epidemiological studies indicating that infection with helminths inversely correlates with metabolic syndrome (104, 105).

THERAPEUTIC MANIPULATION OF DCs FOR THE TREATMENT OF METABOLIC DISORDERS

The ability of DCs to prime strong Th2 responses identifies these cells as an attractive target for therapeutic manipulation of the immune system in the context of metabolic disorders. DCs are widely studied as targets for development of vaccines and immunotherapies because of their capacity to regulate a wide array of T cell responses (106–108). It has been described that DCs accumulate in AT of obese patients and mice (109, 110), and therapeutic manipulation of DCs might also provide a new strategy for targeted treatment of metabolic disorders (**Figure 2**).

In terms of T cell priming, isolated AT $CD11c^{+}F4/80^{low}$ cells from obese mice were shown to preferentially induce Th17 responses (110), and AT $CD11b^{+}$ APCs isolated from insulin-resistant mice promote Th1 polarization (111). However, since both AT macrophages and DCs can express CD11b, CD11c, and F4/80 (109), it is unclear which APC subset is responsible for these effects. In addition, targeting antigen to distinct DC subsets elicits distinct immune responses (112), and therefore, it remains to be determined whether AT DCs would be capable of polarizing Th2 responses *in situ*. These findings highlight the importance of studying AT-associated DC subsets, especially in humans, to identify appropriate subsets for therapeutic manipulation. Furthermore, it has been shown that antigen can efficiently be targeted to and processed by DCs *in vivo* using an antibody against CTL receptor DEC-205 (113, 114), a strategy which may be employed to target AT DCs. Toward this, a DC-restricted receptor on the appropriate subset needs to be identified. Lastly, directing Th2-inducing adjuvants to DCs requires single molecules that can easily be coupled to DC-specific ligands or antibodies. Therefore, proteins such as omega-1 hold promise (38, 39), since they provide a powerful tool to further dissect the molecular mechanisms underlying the induction of a DC-mediated Th2 response. In particular, the identification of the receptors and/or mediators involved in Th2 polarization will provide novel insights for the development of pharmaceutical agents that mimic helminth molecules in their modulation of DCs for Th2 skewing.

CONCLUDING REMARKS

As this review illustrates, helminth molecules can interact with a variety of receptors, that either bind or internalize antigens to condition DCs for Th2 skewing through signaling-dependent and -independent mechanisms. *In vivo*, specific Th2-associated DC subsets are simultaneously exposed to polarizing signals from other immune cells or damaged epithelium. Depending on the helminth species and its migration through the tissue, it is likely that these signals act in concert to ensure robust

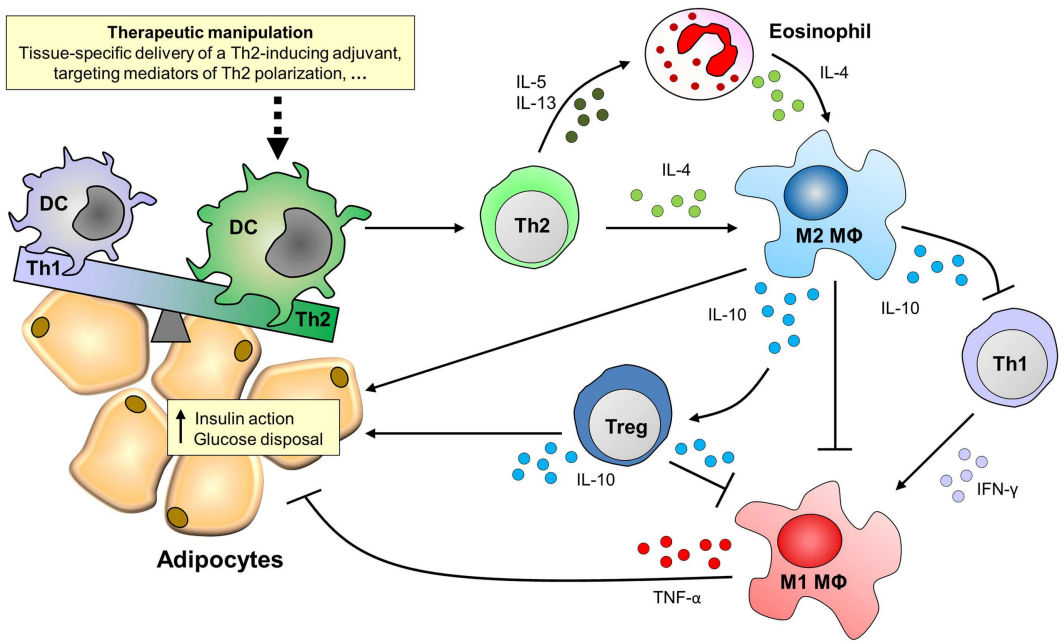

FIGURE 2 | Putative effects of targeting DCs for Th2 polarization on adipose tissue inflammation and insulin sensitivity. Adipose tissues of obese patients and high-fat diet-fed mice are characterized by the accumulation of pro-inflammatory immune cells, like Th1 cells and M1 macrophages, which mediate tissue-specific insulin resistance through secretion of pro-inflammatory cytokines like IFN-γ and TNF-α. By contrast, M2 macrophages that secrete IL-10 protect against insulin resistance via multiple routes. For instance, IL-10 can act directly on adipocytes to potentiate insulin signaling, inhibit Th1 cells and M1 macrophages, and induce regulatory T cells (Tregs), thereby promoting adipose tissue insulin sensitivity and glucose disposal. The maintenance of M2 macrophages in adipose tissue depends on the presence of IL-4, which can be derived from Th2 cells or eosinophils. Novel treatment strategies may therefore focus on therapeutic manipulation of adipose tissue dendritic cells for Th2 polarization.

Th2 polarization, although there seems to be some redundancy. It is now recognized that type 2 immune responses can also regulate energy metabolism, and studying how helminths generate Th2 responses will not only shed light on the mechanisms that promote control of parasite infection and wound healing but may also identify pathways that contribute to metabolic homeostasis.

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The differentiation of CD4$^+$ T-helper cell subsets in the context of helminth parasite infection

***Tiffany Bouchery*[1], *Ryan Kyle*[1], *Franca Ronchese*[1] *and Graham Le Gros*[1,2]*

[1] *Malaghan Institute of Medical Research, Wellington, New Zealand*
[2] *Victoria University of Wellington, Wellington, New Zealand*

Edited by:
Dragana Jankovic, National Institutes of Health, USA

Reviewed by:
John J. Miles, Queensland Institute of Medical Research, Australia
Booki Min, Cleveland Clinic, USA

***Correspondence:**
Graham Le Gros, Malaghan Institute of Medical Research, Victoria University, PO Box 7060, Gate 7, Kelburn Parade, Wellington 6242, Wellington, New Zealand
e-mail: glegros@malahan.org.nz

Helminths are credited with being the major selective force driving the evolution of the so-called "type 2" immune responses in vertebrate animals, with their size and infection strategies presenting unique challenges to the immune system. Originally, type 2 immune responses were defined by the presence and activities of the CD4$^+$ T-helper 2 subset producing the canonical cytokines IL-4, IL-5, and IL-13. This picture is now being challenged by the discovery of a more complex pattern of CD4$^+$ T-helper cell subsets that appear during infection, including Tregs, Th17, Tfh, and more recently, Th22, Th9, and ThGM. In addition, a clearer view of the mechanisms by which helminths and their products selectively prime the CD4$^+$ T-cell subsets is emerging. In this review, we have focused on recent data concerning the selective priming, differentiation, and functional role of CD4$^+$ T-helper cell subsets in the context of helminth infection. We argue for a re-evaluation of the original Th2 paradigm and discuss how the observed plasticity of the T-helper subsets may enable the parasitized host to achieve an appropriate compromise between elimination, tissue repair, containment, and pathology.

Keywords: CD4 T cells, helminth, differentiation, Th2, Th9, Th17, TfH

INTRODUCTION

Helminth parasites are an extremely successful group of organisms infecting over one billion people, with some able to parasitize a host for several decades. Helminths are phylogenetically diverse, with a broad range of migration patterns and life cycles, and are spread across three phyla: nematodes, trematodes, and cestodes. Despite their diversity, the mammalian immune response against these helminths is consistently of the type 2 phenotype characterized by IgE antibody production, eosinophilia, mastocytosis, and specific forms of fibrotic wound repair under the control of the cytokines interleukin-4 (IL-4), IL-5, and IL-13. More recently, the ongoing refinement of our understanding of the type 2 immune response and the recent description of new T-helper cell subsets, force us to re-evaluate the guiding paradigm that would be informative to future studies of type 2 responses in the context of helminth infection.

ROLE OF TYPE 2 IMMUNITY IN HELMINTH INFECTION

The role of type 2 immune responses in immunity against helminths was initially revealed in studies that observed an inverse correlation between levels of parasitemia and the expression of the Th2 cell-derived cytokine IL-4 against the nematode *Trichinella spiralis* (1, 2). These ideas have been further developed in experimental models that show that signaling through IL-4Rα or the IL-4 signaling pathway STAT6 can play important roles in expulsion of, or protection against, the nematodes *Heligmosomoides polygyrus* (3), *Nippostrongylus brasiliensis* (4, 5), *Trichuris muris* (6), the trematode *Schistosoma mansoni* (7), and the cestode *Mesocestoides corti* (8). Although the helminth infection-induced immune effector response normally associated with IL-4 is the production of IgE antibody, the *Trichinella spiralis* experimental infection model is the only one to show a requirement for IgE in protection (9). It should also be noted that IL-4 mediated responses may not always be protective as seen in the study showing STAT6$^{-/-}$ mice have greater resistance to the cestode *Tenia crassiceps* (10).

IL-4 is not the only Th2 derived cytokine that can signal through STAT6. The type 2 cytokine IL-13 has been shown to play a key protective role in many helminth infections, particularly in the expulsion of parasites from the gut by mediating goblet cell mucous production and smooth muscle cell contraction sometimes referred to as the "weep and sweep effect" (11). Macrophages express IL-4Rα, and signaling via both IL-4 and IL-13 can induce an alternately activated phenotype. Alternately activated macrophages produce factors that contribute to the repair of tissues damaged by infection (12); they have also been shown to be required for protective responses against some nematode infections (13).

IL-5 is the third cytokine commonly associated with type 2 immune responses and the Th2 cell subset specifically. The main function of this cytokine is the expansion of eosinophils from the bone marrow (14) with overexpression of IL-5 leading to decreased larvae numbers in primary infections of the nematodes *N. brasiliensis* and *Angiostrongylus cantonensis* (15). Genetic deletion or antibody neutralization of IL-5 or the IL-5 receptor α (IL-5Rα) show a requirement for IL-5 and eosinophils in protective immunity against secondary infections of *Strongyloides stercoralis*, *Strongyloides venezuelensis*, and *Onchocerca lienalis* (16). Eosinophils and IL-5 have also been shown to play an important role in vaccine-induced protection against *Litosomoides sigmodontis* (17).

Recent work has reported that the Th2 cell population is heterogeneous, containing some subpopulations of Th2 cells that produce both IL-5 and IL-13 in the absence of concomitant IL-4 expression (18) and also some subpopulations of Th2 cells that are IL-5$^+$ or IL5$^-$, while expressing IL-4 (19). Furthermore, a study by Liang et al. demonstrated that production of IL-4 and IL-13 is spatially separated with IL-13 being poorly expressed at low levels by lymph node (LN) CD4$^+$ T-cells but strongly expressed by CD4$^+$ T-cells found in the lung (20). These data, along with others, showing that LN CD4$^+$ T-cells expressing IL-4 in response to *H. polygyrus* are primarily of the Tfh phenotype (T follicular helper) (21), negates the view that IL-4 production is a sufficiently comprehensive marker for all the T-helper cell subsets activated during the full expression of a type 2 immune response. It also raises the issue of how we need to have a broader view of how CD4$^+$ T-helper cells should be defined and identified as contributors to type 2 immunity.

THE PRIMING OF TYPE 2 T-CELLS IN HELMINTH INFECTION

The cellular and molecular mechanisms that lead to the priming of type 2 T-cells during helminth infections are not well understood. The IL-4 producing Th2 cell has received the most attention in this regard, with early expression of *Il4* used as a marker of pre-commitment to a T-helper cell of the type 2 lineage. Although IL-4 has been clearly demonstrated to promote overwhelming polarization and differentiation of naïve CD4$^+$ T-cells into Th2 *in vitro* (22), it has been difficult to identify the *in vivo* sources of IL-4 that are able to affect the initial Th2 cell priming. More importantly, *in vivo* studies indicate that Th2 cells can be effectively primed even in the absence of IL-4- and STAT6-dependent signaling (23–25), thus suggesting that signals other than IL-4 must be operating physiologically. The difficulty in identifying such signals has led to the formulation of a number of models of Th2 priming, which are briefly outlined in **Figure 1** below, and linked to available evidence in helminth infection models. Some of this evidence has recently been reviewed (26) and thus is only briefly discussed here.

Several lines of experimental evidence suggest that low-avidity interactions between T-cells and antigen presenting cells (APC) favor the development of Th2 immune responses (27) (**Figure 1A**). Recent work in support of this possibility includes studies of differential activation of T-cells using antigens of varying affinities or concentrations (28, 29), different T-cell–APC ratios (30) and, more recently, the reduced APC-naïve T-cell contact time observed when T-cells are primed *in vivo* in conditions that favor type 2 rather than type 1 differentiation (31). This model of Th2 priming is strongly supported by many elegant studies in well-defined experimental systems, often using homogeneous T-cell populations expressing clonal T-cell receptor (TCR). However, models in which T-cell avidity is the sole driver of T-helper

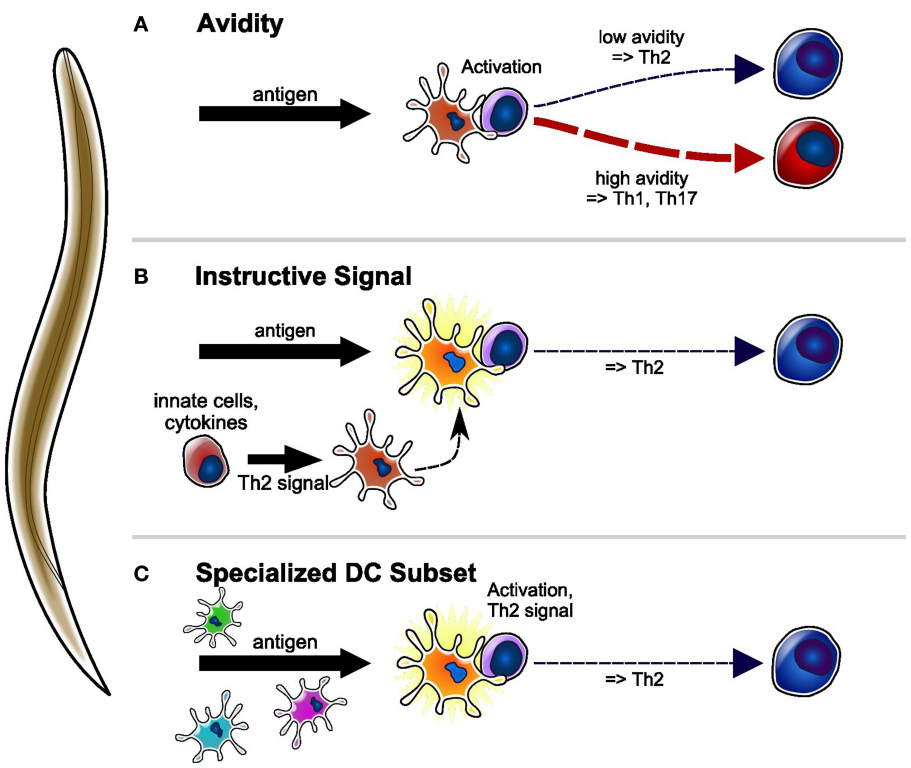

FIGURE 1 | Proposed models of Th2 differentiation induced by helminth parasite antigens presented by DC. (A) Antigen taken up by DC is presented to specific CD4 + T-cells. Low-avidity interactions between CD4 + T-cell and DC result in the priming of Th2 cells, whereas high avidity interactions result in the priming of Th1 or Th17 cells. **(B)** DC conditioned by parasite-induced innate cytokines/cells, or directly conditioned by parasite products, acquire the ability to prime Th2 responses. Th1 and/or Th17 responses are initiated by DC conditioned by other innate signals. **(C)** A specific DC subset that is uniquely able to take up parasite material is programed to prime Th2 immune responses. The priming of other T-cell phenotypes requires other DC subsets.

cell differentiation are difficult to reconcile with the observed prevalence of specific phenotypes of immune response during infections, when complex mixtures of antigens interact with T-cells of a range of specificities such as those found in polyclonal repertoires (32). The wide range of T-cell avidities involved in such situations would predict that the resulting immune response should include a mixture of T-cell phenotypes, but this is not normally observed. It is possible that APC-derived signals, which are differentially invoked by infectious agents such as intracellular versus helminth parasites, might be involved in modulating the strength of $CD4^+$ T-cell activation, thereby resulting in preferential differentiation of a certain T-helper subset (33–36). At this stage, the precise contribution of the avidity of APC-T-cell interaction to the induction of Th2 immune responses to helminth parasites remains to be determined.

An alternative possibility is that Th2 cells might arise as a "default," in situations where the cytokines that normally direct T-cells to differentiate into other phenotypes, such as Th1 or Th17, are absent. Indeed, although parasites still require co-stimulation in order to induce optimal Th2 priming (37, 38), they mostly lack the microbes-associated molecular patterns (MAMPs) that are key to strong APC activation, co-stimulatory molecule expression, and IL-12 secretion (39–41). Alternatively, low-avidity APC-T-cell interactions may fail to sustain the CD40L expression necessary for optimal IL-12 production, as is observed with antigen-MHCII ligands that engage the TCR with low avidity. While this scenario could be consistent with the low-avidity model discussed above, it is not consistent with experiments in IL-12 KO mice showing that lack of this cytokine does not result in activation of Th2 responses (42), or with many experiments showing that helminth products suppress IL-12 production (43, 44) and instruct DC to initiate Th2 immune responses (39, 45–49). Thus, the overall evidence in support of this model remains limited.

Third, Th2 immune responses may be initiated by instructive signals from the APC. These signals may be acquired by APC through interactions with innate cell populations or mediators, or may be constitutively expressed by specialized subsets of APC. The nature of the APC that prime Th2 responses has been debated, with mast cells (50), B-cells (51), and basophils (52) being suggested as necessary, or even sufficient (53, 54), for *in vivo* Th2 induction in various models of immune responses including helminth infections. However, later experiments in a *S. mansoni* model provided clear evidence that DC depletion was sufficient to ablate Th2 induction, while basophil depletion had no effect (55). While it may be reasonable to hypothesize that parasites with different life cycles and target tissues may also involve different APC populations, a key role of DC in priming Th2 responses is consistent with a wealth of experiments in other types of T-cell responses, and also with older experiments in B-cell-deficient mice (56, 57). Altogether, the weight of experimental evidence appears to support DC as being the primary, and possibly the sole, initiator of Th2 immune responses. This conclusion then leads to the question: if DC is the principal APC population that primes Th2 responses, which properties enable them to do so, and how are these properties acquired?

The interaction of DC with helminth products has been extensively examined using DC generated in culture or, less frequently, *ex vivo* spleen DC. These experiments were highly informative and revealed, among other things, the limited upregulation of co-stimulatory molecules on DC by helminth products (36, 40), the role of CD40 (58, 59), OX40L (60), IL-4, and IL-12 (58), and the effects of treatment with TLR ligands (61). However, these results are limited by the fact that the cultured DC used in many of those experiments may not have a physiological equivalent *in vivo*, and splenic DC are not necessarily involved in the immune response to the helminths of interest. These results must, therefore, be extended and confirmed using *in vivo* models, which involve relevant antigens, DC subsets, and routes of exposure.

As discussed above, helminths and their products can directly or indirectly condition DC to prime Th2 responses (**Figure 1B**). On the basis of current evidence, the best candidate molecule associated with DC's ability to program responding $CD4^+$ T-cells to a Th2 phenotype is OX40L (62, 63). Blocking OX40L on human DC also suppresses their ability to prime IL-4-, IL-5-, and IL-13-producing T-cells *in vitro* (64); however, studies using transfer of mouse DC conditioned *in vitro* using SEA (*S. mansoni* Soluble Egg Antigen) suggest that OX40L may also control T-cell expansion *in vivo* (60). Expression of OX40L on DC can be induced by the innate cytokines TSLP (Thymic Stromal Lymphopoietin), IL-25, and IL-33 (64–66), which can be released by damaged epithelia. In addition, some parasite products (39, 67) and CD40 signaling can also cause upregulation of OX40L expression (60, 67). Accordingly, innate cytokines were found to be dispensable for IL-4 production after infection with several – but not all – helminths, suggesting that parasite products may be able to bypass the requirement for innate cytokines (49, 68, 69). Perhaps more surprisingly, it was also found that $OX40^{-/-}$ mice can generate IL-4 responses to *N. brasiliensis* (70), whereas IL-4 responses to *H. polygyrus* are reduced but not ablated, suggesting a variable ability of helminth parasites to bypass or replace the requirement for OX40L co-stimulation. Our experiments comparing Th2 immune responses induced by various agents suggest that helminths may not be unique in their ability to bypass the TSLP/OX40L axis, and that HDM given intradermally can also induce TSLP-independent Th2 responses (48, 71). These results may suggest that route of exposure has a substantial effect on the involvement of innate cytokines in the resulting Th2 immune response. While this possibility is plausible, it must be reconciled with information on the innate environment in different tissues and, most importantly, must be addressed and confirmed experimentally.

Finally, Th2 responses may require a specialized DC subset that is specifically programed to carry out this function (**Figure 1C**). The concept of a Th2-dedicated DC subset is not new (72), and may fit with the DC heterogeneity that is gradually being revealed in all tissues. In line with this notion, recent publications identified a subset of skin DC, expressing the carbohydrate-binding molecule CD301b (73) together with PD-L2 (Programed cell death ligand 2), which is preferentially able to take up "Th2-inducing" antigens. These $CD301b^+$ DC required IRF4 (interferon regulatory factor 4) for their development, and were necessary for the priming of IL-4- and IL-13-producing T-cells *in vivo* and *in vitro* (74–76), but not for IL-4-producing Tfh. Interestingly, while essential, purified $CD301b^+$ DC were unable to prime Th2 responses *in vitro* or upon transfer into recipient mice, suggesting that another cell

population was also required (75). Similar to those reports, our studies examining the DC populations involved in the immune response to non-viable *N. brasiliensis* larvae given subcutaneously (48) found that parasite material was preferentially taken up by a migratory population of $CD11b^+$ DC also expressing CD301b, PD-L2, and IRF4. However, unlike the studies above, we were able to show that transfer of total migratory DC from mice exposed to non-viable *N. brasiliensis* larvae could prime Th2 responses in naïve mice, and that this property was independent of the antigen specificity of the responding T-cells. Importantly, we also found that DC from untreated mice could induce T-cell expansion *in vivo*, but not Th2 responses. Thus, our results suggest that exposure to helminths and the attending innate signals are important factors in conditioning DC for Th2 priming. As our experiments used transfer of mixed populations of DC, we cannot conclude on whether Th2 priming was the property of one specific DC population, or whether it required the cooperation of several DC subsets. In any case, the powerful Th2-inducing properties of helminth parasites are likely to provide a useful model in which to investigate functional DC subsets in airway and intestinal tract, and their relationship to $CD301b^+$ DC. The report that IRF4 expression by DC is necessary for the priming of Th2 responses to inhaled allergens (77) suggests that DC populations able to prime Th2 responses in different tissues may share some common features. Whether these observations also apply to the other $CD4^+$ T-helper cell subsets has yet to be determined.

NEW T-HELPER CELL SUBTYPES ASSOCIATED WITH HELMINTH INFECTION

The development of new technologies, including multicolor flow cytometry and the engineering of fate-mapping and cytokine reporter mice, has lead to the discovery and definition of new subsets of T-helper cells in the past 10 years, namely Th17, Th22, Th9, Tfh and the recently suggested ThGM (T-helper producing GM-CSF). The roles of these newly described subsets, especially in the context of helminth infection, have not been fully elucidated. Here, we review the findings to date in this area, and outline the future questions that will be important to address. The role of Tregs in helminthes infection has been extensively reviewed (78–80) recently and so will not be discussed in this review.

Th9 CELL SUBSET IN HELMINTH INFECTION

IL-9 was originally associated with the Th2 immune response, with reports that IL-9 expression by $CD4^+$ T-cells was high in Th2-pre-disposed, susceptible BALB/c mice infected with *Leishmania major*, and lower in resistant C57BL/6 (81). This view was further confirmed in anti-helminth immunity a few years later (21, 82, 83). However, recent work has shown that IL-9 and IL-4 are rarely produced by the same T-cells, thus suggesting that IL-9-expressing cells represent a discrete T-helper subset, termed Th9 (84, 85). However, both Th17 and iTregs cells have also been shown to be able to produce detectable amounts of IL-9, though not to the extent of Th9 cells. The status of Th9 as a T-helper subset has been further strengthened by the discovery that IL-4 and TGF-β were permissive for Th9 subset differentiation (84) with PU.1 defined as the necessary transcription factor. It is important to note that even if Th9 is now considered as a distinct subset, its proximity to cells of the Th2 subset is re-enforced by both the demonstration that IL-4 is needed to differentiate Th0 cells into Th9, and by the observation of inter-conversion of Th2 into Th9 in presence of TGF-β (84). More recently, however, IL-1 family members have been shown to be able to trigger an IL-4-independent Th9 differentiation (86).

IL-9's role in helminth infection has recently been suggested in two consecutive studies showing that IL-9 transgenic mice infected with either *T. muris* or *T. spiralis* had an increased Th2 response and faster expulsion of the parasite from the intestine (82, 83). In these studies, increased mast cell and eosinophil numbers correlated with increased IL-9 levels, and were suggested as downstream cellular effectors. However, further studies showed that mice vaccinated with IL-9-OVA complex recruited similar numbers of mast cells and eosinophils to the gut of *T. muris*-infected mice, even though the treatment inhibited expulsion of the parasite (87). No other change to the type 2 response was noted. Conversely, vaccination with IL-9-OVA complex did facilitate expulsion of *T. spiralis*, illustrating that despite the general association of type 2 immunity with helminths, the effectiveness of each subtype is fine-tuned to the parasitic species involved (88). In this regard IL-9 has been shown to increase jejunal muscle contractility, and in IL-9-OVA complex vaccinated mice infected with *T. muris* contractility of the intestine was significantly decreased (88).

More recently, IL-9 has been shown to be produced by T-cells during *N. brasiliensis* infection (89, 90) with adoptive transfer of Th9 cells shown to be sufficient for mediating worm expulsion (89). However, the modest differences in worm burden detected between infected $IL\text{-}9^{-/-}$ and wild type mice, the high experimental variability, and the need for timing differences for expulsion to be considered indicate that further work is needed to determine the role of IL-9 in the context of immunity to reinfection (90).

In a *Trichuris* model, using CD4dnTGFbRII mice (which lack Th9 cells) evidence suggested that Th9 cells are required for efficient expulsion of the parasite (84). The susceptible phenotype was associated with a decrease in mastocytosis and IL-9 expression in the mesenteric LN. However, the presence and frequencies of IL-9 producing $CD4^+$ T-cells *in vivo* was not assessed in this study and the CD4dnTGFbRII mice had a decreased IL-4 response but normal IL-13 response to *Trichuris*, indicating possible defects other than the lack of IL-9, that could contribute to susceptibility. Finally, IL-9 has a role in controlling fibrosis through upregulation of prostaglandin E2 (PGE2), a well-known anti-fibrotic molecule (91), and has been recently shown to be essential to mucosal wound healing in an oxazolone-induced colitis model, through the upregulation of claudin-2 in intestinal epithelial cells (92).

Th17 CELL IN HELMINTH INFECTION

Th17 was identified as a subset distinct from Th1 and Th2 differentiation in 2005 (93), based on cellular production of IL-17 in the absence of IFN-γ or IL-4. These cells are considered proinflammatory as they express high levels of their signature cytokine IL-17, as well as IL-22, IL-6, and TGF-β, all under the control of the master transcription factor RORγt. Combinations of IL-23, TGF-β, IL-6, and IL-21 direct the differentiation of Th17 cells from naïve $CD4^+$ T-cells (94). Th17 cells exacerbate experimental

autoimmune encephalomyelitis (EAE) (95) but also contribute to protection against models of fungal infection (96).

The role of Th17 in helminth infection has principally been studied in *S. mansoni* models, where it has been strongly associated with infection-induced immunopathology. The pathologic role of IL-17 in helminth infection was originally recognized by its association with the development of hepatointestinal perioval granulomas caused by *S. mansoni* infection. In these early studies, $CD4^+$ T-cells were known to be required for the development of the pathology (97, 98), and under the Th1:Th2 paradigm, the role of IL-17 was interpreted as being part of the Th1 immune response causing increased pathology versus a less destructive Th2-dominant response (99).

Further, in an interesting study of mouse strain related susceptibility to pathology (100), it was found that pathology was diminished in IL-12p40$^{-/-}$ mice but not IL-12p35$^{-/-}$ and that IL-17 but not IFN-γ levels correlated with disease, indicated that pathology was likely controlled by Th17 cells in an IL-23 dependent manner (100). Further evidence for this was provided by genetic depletion of IL-23 and disruption of IL-1β signaling leading to decreased IL-17 levels and decreased pathology (101). Furthermore, $CD4^+$ T-cells from TCR transgenic mice recognizing *Schistosoma* antigen Sm-p40 expressed IL-17 when stimulated by DCs loaded with *Schistosome* eggs (102). Antibody neutralization of TGF-β lead to decreased plasma levels of IL-17 and a reduced worm burden, although this may have also changed other parameters including Treg populations (103). IL-17 from RORγt expressing Th17 cells was also associated with the severe pathology seen in natural infection with *Schistosoma japonicum* with antibody neutralization of IL-17 leading to diminished neutrophil infiltration in the liver and reduced hepatic and pulmonary pathologies (104–106). IL-17-associated pathology is also evident in human studies with children infected with *Schistosoma haematobium* having a higher circulating Th17:Treg cell ratio than those children infected but pathology-free, mirroring the ratios seen in high-pathology CBA mice compared to mild pathology C57Bl/6 (107). While studies identifying T-cells producing IL-17 in *Schistosoma* infected tissues show that most are $CD4^+$ T-cells, the link to the expression of the transcription factor RORγt has been rarely attempted, also the downstream mechanisms of IL-17-associated immunopathology remain largely unknown, with few studies indicating which responding cellular components mediate granulomatous damage.

With respect to immune responses to other helminth phyla, the role of Th17 is less clear. An association between pathology and Th17 has been suggested in human filarial infection with patients exhibiting lymphedema caused by lymphatic filariasis having increased numbers of peripheral blood lymphocytes producing IL-17 along with decreased Tregs number (108). The presence of cytokines IL-1β, IL-23, and TGF-β have also been shown to augment these filarial-specific Th17 responses (109). Pulmonary hemorrhaging and neutrophilia caused by migration through the lung by *N. brasiliensis*, a rodent hookworm, was also shown to be dependent on IL-17 expression (110).

While the association of Th17 and IL-17 with pathology in helminth infection is robust, there is limited evidence of a role of Th17 in protection against helminths. One study, looking in blood cultures from patients who received praziquantel to clear *S. haematobium* infection demonstrated an association between high levels of Th17-associated cytokines (IL-21 and IL-23) with a decreased risk of re-infection (111). IL-17 expression has also been linked to both mucosal damage and hyper-contractility of the jejunum of *T. spiralis*-infected mice suggesting a role of Th17 in expulsion of the worms from the gut, but the study is highly preliminary and no depletion of IL-17 was attempted (112). *Echinostoma caproni* establishes a chronic infection in mice while rats are able to expel the worms after 4 weeks post-infection. Intestinal Th17-family cytokines IL-17, IL-23, and TGF-β were markedly upregulated in rats but not in mice, suggesting Th17 activation may be protective in this model (113). Conversely, an ovine model of *Teladorsagia circumcincta* infection demonstrated increased Th17 cytokines correlating with susceptibility to infection (114). Overall, the role of IL-17 producing Th17 cells in helminth driven immune responses is preliminary, and further work is needed.

Th22 CELL IN HELMINTH INFECTION

The cytokine IL-22 is normally associated with responses to microbes and its production mainly attributed to Th17 cells in both mice and human beings (115). However, a distinct subset of human skin $CD4^+$ T-cells has recently been shown to produce IL-22 but not IL-17 or IFN-g (116–118), and thus has been given the term "Th22". Th22 responses have been more widely studied in human beings than in mice so far, with a broad range of functional activities demonstrated, both proinflammatory and anti-inflammatory. While IL-22 is mainly produced by immune cells, the expression of its receptor IL-22R is mostly restricted to non-hematopoietic cells, such as epithelial cells (119). Th22 cells arise from the stimulation of naive T-cells in the presence of IL-6 and TNFα or presentation of antigen in the context of plasmacytoid dendritic cells, and appears to be independent of RORγt but dependent upon the aryl hydrocarbon receptor (AHR) (116, 120, 121).

To date, only a few studies have attempted to address the role of IL-22 in the context of helminth infection. IL-22 is upregulated in the intestinal mucosa after infection by *Trichuris trichuria* or *Necator americanus* in human beings (122–124) and Th22 frequency in PBMC is higher in filarial-infected patients than in healthy controls (109). While helminth infection clearly induces IL-22, so far no role for Th22 in either immune-mediated protection or pathology has been proven. In fact, IL-22$^{-/-}$ mice infected with *S. mansoni* did not present significantly modified immune responses compared to wild type controls, neither did the absence of IL-22 modify the establishment of the parasite or the development of pathology (125). In filarial infection, Th22 frequency in PBMC was higher in lymphedema-positive people than in asymptomatic people, as was their frequency after antigen restimulation with both adult and microfilarial stages of the parasite (109). As reported in the above section, IL22 produced by Th17 cells plays a role in gut expulsion of *N. brasiliensis* and *T. muris* (126).

Th22 has also been reported to be involved in skin repair mechanisms and as such may be relevant to the pathology following skin penetration by helminths. Furthermore, IL-22 is known to have a role in the control of dysbiosis in the gut (127). As helminths have co-evolved with both the host and its microbiome (128), in order to observe the role of Th22 in helminth

infection, it may be required to study the tripartite interaction of microbiome–macrobiome–host rather that the classical bipartite helminth–host interaction.

Tfh CELL SUBSET IN HELMINTH INFECTION

In distinction to the other $CD4^+$ T-helper cell subsets, Tfh cells were not initially described based on cytokine production and transcription factor expression patterns, but rather by the expression of the surface marker $CXCR5^+$ denoting its localization to the germinal center of human tonsils. Tfh have since been shown to promote germinal center formation and class switching of B-cells in mice and are further characterized by their expression of the inducible co-stimulatory molecule (ICOS), the inhibitory receptor programed cell death-1 (PD-1), and B and T lymphocyte attenuator (BLTA) (129). Bcl6 has been identified as the master transcription regulator for Tfh cells (130–132).

In the context of *N. brasiliensis* infection, Tfh cells have been demonstrated to express IL-4 but not IL-13 (20). This study also reports that these IL-4^+ Tfh cells localize to the B-cells follicle, and not to tissue sites such as the lungs. Interestingly, work by Glatman Zaretsky et al. demonstrated that IL-4-expressing $CXCR5^+$ Tfh cells could develop from adoptively transferred Th2 cells ($CXCR5^-$ and $PD1^-$) in B-cell-sufficient hosts during *S. mansoni* infection (133). Furthermore, IL-4 production by Tfh cells is essential for proper B-cell expansion and activation, as demonstrated by a reduction in B-cell activation in IL-$4R^{-/-}$ mice (21).

The role of Tfh was studied in the context of *S. japonicum* induced pathology model (134). In $ICOSL^{-/-}$ mice that are deficient in Tfh cells, a diminution of the liver pathology is associated with a decrease in Th1 and Tregs, whereas Th2 and Th17 are unaffected. Furthermore, adoptive transfer of Tfh cells to $ICOSL^{-/-}$ mice proved sufficient to re-establish pathology characterized by the accumulation of cells in granulomas in the liver (134). Furthermore, plasma cells have been shown to be present in the granuloma induced by *S. japonicum* infection in both pig and mice, and depletion of B-cells reduced pathology in this model (135, 136).

In the context of non-helminth infections, Tfh cells have been shown to produce IFN-γ and several studies suggest that Tfh cell production of Th1-, Th2-, and Th17-associated cytokines provides further evidence that they are potentially derived from these lineages (129). Whether these different patterns of cytokine expression reflect different subsets of Tfh cells, akin to those observed in T-helper subsets and ILCs, is still unclear. The use of Bcl6 reporter mice may help to distinguish Tfh cells from other T-helper subsets and to provide an answer to this question, and may allow the identification of other cytokine patterns in Tfh cells that are induced in helminth infection in parallel with the other T-helper subsets.

HYPOTHETICAL ThGM CELL SUBSET IN HELMINTH INFECTION?

ThGM are the most recently proposed $CD4^+$ T-helper subset having been described in *in vitro* studies developing from naïve $CD4^+$ T-cells stimulated with anti-CD3 and anti-CD28, in the absence of IL-4, IFN-γ, and IL-12 (137). It is important to note that to our knowledge, this subset has not been described *in vivo* so far and that its existence will have to be further confirmed.

Putative ThGM cells produce high levels of GM-CSF, while not producing Th1- or Th2-associated cytokines. The authors further show that they do not express T-bet, GATA3, RorγT, or Foxp3, thus supporting the idea that they indeed constitute a new $CD4^+$ T-helper subset (137). GM-CSF is a pluripotent cytokine, which has been shown to induce T-cells proliferation and activate macrophages and neutrophils, among other cells, and the absence of this cytokine has been shown to negatively impact the differentiation of both Th1 and Th2 responses (138).

Contrasting roles have been shown for GM-CSF in helminth infection settings. In *N. brasiliensis* infection, mice deficient in GM-CSF show no reduction in worm burden in the lungs or gut in both primary and secondary infection when compared to wild type mice (139).

In *Onchocerca volvulus* infection, presumably immune individuals (negative for the parasite, but living in endemic area) have a mixed Th1/Th2 response to L3 and microfilariae antigen, contrary to infected individuals that present only a strong Th2 response to those antigens. In particular, GM-CSF, at that time considered as a Th1 cytokine, was greatly enhanced in the putatively immune individuals (140). Co-cultivating human PBMCs *in vitro* with the *Schistosoma* antigen SmGST28 has been shown to be sufficient to induce some granulomatous formations, and GM-CSF is needed for this reaction (141), suggesting a role for this cytokine in development of pathology.

FURTHER T-HELPER CELL DIVERSITY AND PLASTICITY IN HELMINTH-INDUCED TYPE 2 IMMUNE RESPONSES

In the original Th1/Th2 paradigm, it was proposed that the T-helper cell subsets were distinct and negatively regulated each others' activities, all underpinned by regulatory epigenetic methylation signatures interacting at IFN-γ, *IL4*, Gata-3, and T-bet gene loci (142, 143). However, this concept has to be revised in light of the recent discoveries of additional functionally diverse T-helper subsets including those with mixed Th1/Th2 signature cytokine phenotypes and by the observation that certain T-helper subsets can reverse their degree of polarization (144, 145).

Although it has been known for some time that double-positive IFN-γ^+IL-4^+ T-cells can be detected in experimental models of Th2 differentiation (146), when viewed in the context of the original Th1/Th2 paradigm, they were considered to be Th0 cells that were not yet committed to a polarized phenotype. However, several recent publications clearly demonstrated that these Th1/Th2 hybrid cells are stable both *in vitro* and *in vivo* after infection with either the trematode *S. mansoni* and the nematode *H. polygyrus* helminth infections (146, 147). Furthermore, this Th1/Th2 hybrid cells arise in a IL-18-dependent manner in *S. mansoni* infected mice (148). Interestingly, adoptive transfer of Th1/Th2 hybrid into Th1 or Th2 inflammatory models (LCMV and allergic airway inflammation) showed in both cases a reduced pathology associated with the inflammation (147). At the molecular level, these Th1/Th2 hybrid cells present with an intermediate expression of Gata-3 and T-bet as compared to Th2 and Th1 cells, respectively, due to an intermediate signature of methylation, for example,

gata3 methylation was 36% in those cells, versus 60% in Th1 and 8% of Th2 (149).

Both T-bet and GATA3 can regulate each others expression (150), and in a recent study, RORγt and Foxp3 were shown to directly interact in a way that determines Th17 versus induced Treg lineage (151, 152). Also, it has been shown that Tfh cells can express both Gata3 or T-bet and that while Bcl6 decreases their relative expression it does not block it completely (153). Taking these observations together, it would seem reasonable to speculate that it is the ratio of transcription factors induced that may determine the fate of any developing T-helper subset and that as a T-helper cell differentiates there is available a broad contiguous range of gene expression patterns for shaping its ultimate phenotype during an immune response against a helminth (**Figure 2A**) (154).

The possibility of potential inter-conversions of different T-helper subsets in the context of a helminth infection has recently been studied (155). Both *in vitro*- and *ex vivo*-generated Th1 and Th17 cells, adoptively transferred into mice later infected with *N. brasiliensis*, were shown to convert into IL-4 producers while losing their own signature cytokine expression. Also, both the iTregs and the nTregs were found to be stable *in vivo* in this study, with only low number of cells converting to express IL-4 (155). It is interesting to note that the *in vitro*-generated Th1 and Th17 cells showed a lower propensity to convert after transfer, suggesting that *in vitro* culture has a strong impact on the degree of T-helper cell plasticity that can be observed and perhaps explaining why the initial *in vitro* investigations into T-helper subset differentiation found subsets were highly stable and thus terminally differentiated.

Based on observations of the diversity and apparent plasticity of T-helper subsets phenotypes that can be detected now and on dynamic systems theory, we propose a landscape representation of the possibility of inter-conversions and intermediary states for T-helper cell subsets developing in the context of a helminth-induced immune response (**Figures 2B,C**). We have represented the dynamic of T-cells' fates on a quasi-potential landscape in which the different subsets constitute stable states, also called attractors. In this view, the subsets are in "valley" or "flat" areas. To transit from one stable state to another, the system needs to be perturbed, for example by an infection that would push cells toward certain attractors or away from others. Unstable states are usually represented as "hills." The Th1/Th2 hybrid state is stable enough, but less that the more terminally differentiated Th1 and Th2, and is thus represented by a well with less depth than Th1 or Th2.

Using such a "continuum of T-helper cell phenotypes" paradigm, it would be predicted that in host tissues responding to helminths there would be a gradient of activated and differentiated T-helper cell subsets with the most fully differentiated being stable and having lost much of their plasticity. Such terminally differentiated helper T-cells, maybe such as Th2, probably represent a small proportion of the pool of memory effector T-cells that maintain the helminth antigen specificity and the appropriate cytokine profile.

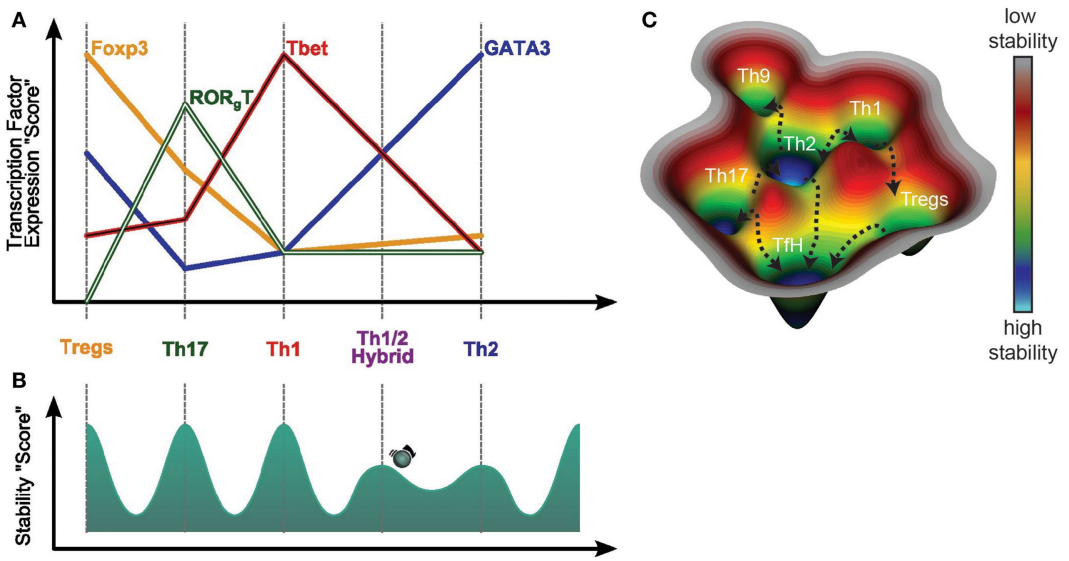

FIGURE 2 | Dynamic of T helper differentiation. **(A)** The dynamic of T helper differentiation can be visualized as a "potential landscape" in which each T helper subset represent a stable position or "valley" and the transition from one subset to another, would be a "hill", difficult to pass. Initially, the transition state between T helper subsets was considered as instable, and thus not observable *in vivo*. However, Th1/Th2 hybrid population has recently been reported to be stable after helminth infection. As this hybrid state is less abundant than Th1 or Th2, one could presume that the hybrid population is less stable that the Th1 or Th2 subsets, thus represented as a less deep well. **(B)** This transition between subsets can be further defined by the ration of transcription factor participating in the fate determination of each subset. For example, the Th1/Th2 population has been shown to present intermediary level of gata-3 and T-bet expression as compared respectively to Th2 and Th1. Through similar transcriptomic approach, generalised on all the T helper subsets, it would thus be possible to define a ration of transcription factors necessary to enable the switch from one subset to another. **(C)** The plasticity of the T helper subsets is represented in a conceptual 3D potential landscape and illustrate that the diverse repertoire of T helper cell subsets, and its important plasticity, enable the host to have an array of fine-tuned adaptive responses to both control the parasite development and avoid and repair pathology caused by the worm migration.

TYPE 2 IMMUNE RESPONSES TO HELMINTH INFECTION ARE A COMPROMISE BETWEEN PROTECTION, SUSCEPTIBILITY, TISSUE REPAIR, AND PATHOLOGY

The original paradigm explaining resistance or susceptibility to helminths was described as a simple balance between Th1 (the susceptible, pathologic response), and Th2 (the response conferring parasite killing and elimination). However, this paradigm did not satisfactorily explain why so many parasites are able to establish themselves in hosts for extended periods of time (sometimes for decades) without causing any major clinical symptoms, nor why, in endemic environments, hosts are continually being reinfected with no apparent sign of disease. The hypothesis that the immune system would ignore the parasite infection was unconvincing, begging the question of whether these parasites themselves actively downregulate the host immune response and control pathology. Consequently, much research on helminth infection has focused on understanding how the parasite could regulate immune responses and teasing apart what is the physiological purpose of the type 2 effector responses in terms of benefit to host survival.

With the emergence of data showing that helminths and their products could be used to prevent/cure both allergic and autoimmune (156), our understanding of the immune response against helminth has changed to take into account the regulatory mechanisms induced by the parasites. From this emerged a new concept, the "modified Th2," characterized by a decrease IL-5 and IL-13 expression and an increase of anti-inflammatory cytokines, such as TGF-b and IL-10. Other cells types, such as regulatory myeloid cells or regulatory B-cells have been shown to be involved in the downregulation induced by helminth.

The Th2 immune response has also been shown to be part of a wound repair response, with the ability to block a runaway pathology. This is clearly illustrated in IL-$4^{-/-}$ mice infected with *S. mansoni* that die from excess pathology, even if their worm burden was similar to the wild type control mice (157). Th2 immune response has been considered as having evolved to cope both with helminth infection and with damage repair mechanisms, necessary to the survival of the host against those multicellular metazoan parasite migrating through tissue (12, 158). New research has shown that IL-9 has roles in controlling fibrosis and wound repair (91, 92), and that IL-22 from Th22 and Th17 cells promotes healing by increasing proliferation and survival of keratinocytes and intestinal epithelial cells (118, 159, 160). These data indicate that Th2 may not be the only T-helper subset contributing to the repair of helminth-induced tissue damage.

Furthermore, ES-62, a secretory product of the filarial nematode *Acanthocheilonema viteae*, may play with the balance of the different T-helper subsets in order to diminish the protective immune response (161). In a murine model of OVA-induced allergic airways inflammation, ES-62 decreased Th2 responses, as shown by a reduced level of IL-4 in the broncho-alveolar fluid, decrease eosinophils recruitment, and IgE. In parallel, the Th1 immune response is exacerbated, and neutralization of IFN-γ initiated the allergic inflammation blocked by ES-62 treatment. Th17 was also suppressed in this model, and that this suppression is responsible for the increase of the Th1 response. Importantly, ES-62 in this model did not induce an increase of Treg population, thus suggesting that the immunomodulatory effect of the molecule is to modify the balance of $CD4^+$ T-helper subsets. This is the key data to understanding that the regulation of immune responses is not solely controlled by Tregs, but the different $CD4^+$ T-cells subsets themselves antagonize one another.

Considering the fact that all the currently recognized stable $CD4^+$ T-helper phenotypes have been observed during helminth infections, and that these different subsets contribute variously to either protection, wound healing, susceptibility, or immunopathology, we have designed a new model that condenses what is known about the direct contributions of the subsets and their cross-regulation of each other. This model demonstrates our better understanding of the balanced immune responses during helminth infection **Figure 3**.

The early opposition of Th1 and Th2 is still clearly visible as a driving force for the trade-off between host and helminth survival. Th2 and Th9 segregate together to confer resistance, as they share a common activation pathway through IL-4. They are opposed to Th1 and Th17 in regard to pathology and to Th1 and Tregs in regard to susceptibility and chronicity. This model emphasizes that what may be best for the host is a compromise between elimination of the parasite versus containment and also the need for rapid repair of damaged tissue and avoidance of self destructive pathology. This all-encompassing

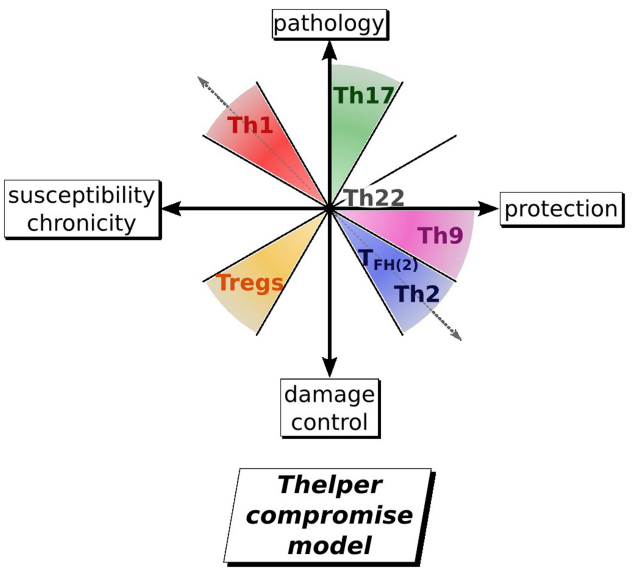

FIGURE 3 | Evolution of the view on T helper involvement in helminth infection. By taking into account all the other T helper subset known to date, it is proposed that an immune response against on helminth can be summarized as a 2D map defined by an axis of susceptibility/protection and an axis of pathology/damage control. For an optimal response against a parasite, the host would thus mount a Th2/Th9 response with a low Tregs response and almost not existing Th1, Th17 response. The Th2 arm of the immune response protects against helminth by expanding ILCs, eosinophils and basophils all involved in parasite expulsion or by activating macrophages in AAM, playing a role in granuloma formation. Th9 rather protects by increasing goblet cells hyperplasia and muscle contractility in the gut. Th17 induced pathology is mainly mediated by neutrophils and inflammatory macrophages. In contrary, Tregs induced development of regulatory macrophages, which control pathology.

view of an immune response gives a better understanding of the host issues at stake and gives a context for further investigations to investigate roles for each of the T-helper subsets. Furthermore, the consideration of a multipartite balance, rather than a one in one balance, would be useful to the design of therapy against helminths (i.e., by developing adjuvant to vaccine that could determine the right balance of Th susbsets to obtain sterilizing immunity, and define the timing to administer such treatment), and to understand how helminth infection or their product could use a therapy against inflammatory and autoimmune disease, caused by the deregulation of different T-helper subsets.

DISCUSSION/REMARKS

The new challenge to the investigation of type 2 immune responses is to determine how many subsets of T-helper cells exist and what mechanisms control the level and degree of plasticity that occurs between T-helper cell subtypes. The question arises as to what would be the benefit to the host to have such complexity and myriad of genetic events underpinning this plasticity of the T-helper cell response. We would argue that the benefit to the host in being able to generate so many T-helper subsets is to have the diversity of options for dealing with the myriad of parasitic forms, invasive routes, and environments that have an endless supply of parasites that invade by physical means. In effect, the diversity and plasticity of the repertoire of functional T-helper cell subsets enable the host to have an array of adaptive responses. While the response might not kill the worm, it will enable the host to repair the more serious damage caused by the migrating parasite, and avoid the fatal consequences of debilitating pathology.

We wish to point out that in order to study the type 2 immune response elicited by helminths, i.e., define the role of the various old and new discrete $CD4^+$ T-cell subsets, both techniques and approaches will have to evolve. For example, as plasticity between different T-helper subsets become increasingly evident, it maybe of interest to define a subset by both the cytokine production/non-production patterns, as well as by the ratio of transcription factors they express. For this matter, engineered reporter mice for particular cytokines and even combinations of cytokines may help in *in vivo* studies but may not reflect the native mRNA or protein. New bioinformatics approaches, such as studies of the transittability (162) (that defines the lower number of molecule switch to go from one fate of differentiation to another one based on a network of molecules involved in shaping the cell fate), could provide in the near future a list of more appropriate marker necessary to define one particular T-helper subset. Moreover, due to the plasticity of the T-cells, we think it is important to remember that immune studies look at dynamic events, and as such looking at a precise time point in the model, may give a wrong picture of the actual mechanism, for example a subset "in transition," may be missed because of the lack of markers used for describing it, or the relative rarity compared with the currently defined stable subsets.

Hopefully, advances in single cell analysis (Fluidigm), sequencing, multiplex quantification of transcripts (such as nCounter, Nanostring that can detect up to 800 genes), advanced multicolor flow cytometry (such as panels up to 20 colors), the emergence of mass cytometry (such as CyTOF that allows multi-detection of up to 34 parameters to date, but could potentially go up to 100), coupled with bioinformatic approaches may offer the new tools necessary for studying the dynamics of T-helper differentiation in the context of helminth infection.

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Antifungal Th immunity: growing up in family

Monica Borghi*[1], *Giorgia Renga*[1], *Matteo Puccetti*[2], *Vasileios Oikonomou*[1], *Melissa Palmieri*[1], *Claudia Galosi*[1], *Andrea Bartoli*[1] *and Luigina Romani*[1]*

[1] *Pathology Section, Department of Experimental Medicine, University of Perugia, Perugia, Italy*
[2] *Polo GGB, Perugia, Italy*

Edited by:
Dragana Jankovic, National Institutes of Health, USA

Reviewed by:
Edward John Collins, The University of North Carolina at Chapel Hill, USA
Cosima T. Baldari, University of Siena, Italy

****Correspondence:***
Luigina Romani, Pathology Section, Department of Experimental Medicine, University of Perugia, Polo Unico Sant'Andrea delle Fratte, Perugia 06132, Italy
e-mail: luigina.romani@unipg.it

Fungal diseases represent an important paradigm in immunology since they can result from either the lack of recognition or over-activation of the inflammatory response. Current understanding of the pathophysiology underlying fungal infections and diseases highlights the multiple cell populations and cell-signaling pathways involved in these conditions. A systems biology approach that integrates investigations of immunity at the systems-level is required to generate novel insights into this complexity and to decipher the dynamics of the host–fungus interaction. It is becoming clear that a three-way interaction between the host, microbiota, and fungi dictates the types of host–fungus relationship. Tryptophan metabolism helps support this interaction, being exploited by the mammalian host and commensals to increase fitness in response to fungi via resistance and tolerance mechanisms of antifungal immunity. The cellular and molecular mechanisms that provide immune homeostasis with the fungal biota and its possible rupture in fungal infections and diseases will be discussed within the expanding role of antifungal Th cell responses.

Keywords: Th cell subsets, immunity, tolerance, fungi

FUNGAL INFECTIONS AND DISEASES IN THE METAGENOMICS ERA: A REAPPRAISAL

Fungi can interact with their hosts (plants, animals, or human beings) in multiple ways, establishing symbiotic, commensal, or pathogenic relationships. Most fungi, such as *Aspergillus fumigatus* and *Cryptococcus neoformans*, and the thermally dimorphic fungi are ubiquitous in the environment, and human beings are exposed by inhaling spores or small yeast cells. In addition, more than 400 species of fungi associated with human beings have been identified (1). In this case, co-evolution of commensals, such as *Pneumocystis jirovecii, Malassezia* spp., and *Candida albicans*, with their mammalian hosts implicates the existence of sophisticated mechanisms to antagonize immunity in order to survive. Once considered pathogenic microbes, the commensal fungal microbiota is now an important component of the human intestinal ecosystem. Indeed, despite the intimate contact of fungi with the human host, fungal diseases in immunocompetent hosts are fairly uncommon, indicating that low-virulence fungi have evolved particular adaptation mechanisms that allow them to persist relatively unnoticed by the immune system (2). This "peaceful" coexistence may digress into overt disease under conditions of immune deregulation, such as in primary immunodeficiency human immunodeficiency virus infection and as a result of immunosuppressive therapies (2). In addition, invasive fungal diseases continue to be a serious problem in patients with hematologic disorders, solid, and hematopoietic organ transplantation as well as in non-high-risk, *sensu strictu*, patients, such as patients with *Mycobacterium tubercolosis* infection, hyper IgE syndrome, and anti-TNF-alpha therapy (3).

The increasing understanding of the importance of the microbiota in shaping the host immune and metabolic activity has rendered fungal interactions with the host and its microbiome more complex than previously appreciated (4) (**Box 1**). Indeed, the complex interactions between fungal and bacterial commensals, either directly or through the participation of the host immune system, all impact on the pathophysiology of a number of inflammatory disease that, in turn, may lead to secondary fungal infections (5, 6). Evidence is accumulating to support the exciting concept that the interaction between different biomes and between the host and the mycobiome are critical in the pathogenesis of fungal infections and other human diseases (1, 7, 8). Here, we will discuss recent findings on host- and microbial-dependent mechanisms of immune homeostasis with the fungal biota and its possible rupture in fungal infections and diseases.

RESISTANCE AND TOLERANCE MECHANISMS OF ANTIFUNGAL IMMUNITY

As the immune system has evolved to accommodate colonization by symbiotic microbes while retaining the capacity to oppose their infectivity, a fine balance between pro- and anti-inflammatory signals is a prerequisite for a stable host/fungal relationship, the disruption of which may lead to pathological consequences. Indeed, despite the occurrence of severe fungal infections in immunocompromised patients, clinical evidence indicates that fungal diseases also occur in the setting of a heightened inflammatory response, in which immunity occurs at the expense of host damage and

Abbreviations: AhR, aryl hydrocarbon receptor; APS-1, autoimmune polyendocrine syndrome type 1 patients; CF, cystic fibrosis; CLR, C-type lectin receptors; CMC, chronic mucocutaneous candidiasis; DCs, dendritic cells; DTH, delayed type hypersensitivity; IDO1, indoleamine 2,3-dioxygenase 1; ILC3, innate lymphoid cells 3; IRIS, immune reconstitution inflammatory syndrome; PRRs, pattern recognition receptors; RVVC, recurrent VVC; Th, T helper; Treg, regulatory T-cells; VVC, human vulvovaginal candidiasis.

Box 1 | The mycobiome at the host/microbiome interface.

The development of culture-independent methods has expanded our knowledge of the mycobiomes found in different body sites, their interface with other biomes, and their association with human health and diseases (1). Alterations in the mycobiome are frequently reported to be associated with various diseases such as cystic fibrosis (9), inflammatory bowel diseases (6, 10, 11), atopic dermatitis (12), or mucocutaneous candidiasis (13). However, it remains to be elucidated whether this variation is primary or secondary to an imbalanced bacterial microbiome. Indeed, interactions of fungi with bacteria *in vitro* have been described [reviewed in Ref. (6)] as well as the clinical relevance of these interactions (14), such as the occurrence of intractable candidiasis in association with antibiotic-induced dysbiosis (15) and of mixed fungal–bacterial species in biofilms (14). Fungal–bacterial interactions can be antagonistic, synergistic, or symbiotic; regardless, they influence the physiological characteristics and survival of either one partner and, consequently, impact on host immune reactivity. Variations in the mycobiome can also be secondary to dysregulated host immune reactivity. The traditional view of a single direction by which bacteria stimulates the immune system, leading to inflammation or autoimmune disorders, has been challenged by a more complex view; the gut immune system does not simply protect from pathogens, but is actively involved in the maintenance of a rich and healthy community of gut bacteria (16). Faults in the immune regulation lead to changes in the bacterial community that in turn feed back into the immune system. Similar to the microbiome, the host/mycobiome interactions also lead to mutual influences. Not only is the host affecting the mycobiome composition and variations, by means of genotype, physiology, immune system, and lifestyle, but also the fungal microbiota may contribute to the balance of inflammation and tolerance at local mucosal surfaces and at distal sites (17).

pathogen eradication (18). A number of fungal diseases are critical examples of such bidirectional influences between infection and immune-related pathology, a condition that highlights the bipolar nature of the inflammatory process in infection. Early inflammation prevents or limits infection, but an uncontrolled response may eventually oppose disease eradication. This conceptual principle is best exemplified by the occurrence of severe fungal infections in patients with chronic granulomatous disease (19), cystic fibrosis (20), or with immune reconstitution inflammatory syndrome (IRIS) (21), an entity characterized by local and systemic inflammatory reactions that can result in quiescent or latent infections manifesting as opportunistic mycoses. Chronic mucocutaneous candidiasis (CMC) and chronic disseminated candidiasis also belongs to the spectrum of fungus-related IRIS (22). Thus, an immune response that limits both fungal infectivity and host collateral damage is required to maintain a homeostatic environment (23). This dual role has recently been accommodated within the conceptual framework of a two-component antifungal immune response, i.e., resistance – the ability to limit fungal burden – and tolerance – the ability to limit the host damage caused by either the immune response or other mechanisms (2). Resistance is meant to reduce pathogen burden through innate and adaptive immune mechanisms, whereas a plethora of tolerance mechanisms, despite less known relative to resistance mechanisms, protect the host from immune- or pathogen-induced damage (24).

MECHANISMS OF ANTIFUNGAL RESISTANCE

Innate immune mechanisms are used by the host to respond to a range of fungal pathogens in an acute and conserved fashion. The constitutive mechanisms of innate defense are present at sites of continuous interaction with fungi and include the barrier function of body surfaces and the mucosal epithelial surfaces of the respiratory, gastrointestinal, and genitourinary tracts. Microbial antagonism, defensins, collectins, and the complement system realize the strict fungus specificity of the constitutive mechanisms and provide opsonic recognition. Multiple cell populations and cell-signaling pathways are involved in the antigen-independent recognition of fungi by PRRs (2, 25). Both murine and human studies have confirmed the association of susceptibility to fungal infections and diseases with genetic deficiency of selected PRRs (2). Because PRRs not only mediate downstream intracellular events related to fungal clearance but also participate in activation of adaptive immunity, deficiencies on innate immune genes also reverberate on the type and quality of the adaptive immune response, including effector CD4+ T helper (Th), regulatory T (Treg), and CD8+ T-cells (2, 25–27).

DENDRITIC CELLS

It is well established that the adaptive immune response, in particular that of T-cells, plays a pivotal role in antifungal host defense (2, 25). Dendritic cells (DCs) play a key role in promoting T-cell differentiation and responses to ubiquitous or commensal fungi. Studies have shown that lung DCs can transport fungal antigens to the draining lymph nodes (28, 29), where they orchestrate T-cell activation and differentiation into effector cells. Through elaboration of distinct sets of cytokines and other mediators, DCs have the unique ability to elicit a robust T-cell response that can be either tolerogenic or pro-inflammatory in nature, based on anatomical location and local metabolic environment. The whole-genome transcriptional analysis of DCs stimulated with fungi evidenced the presence of peculiar transcriptional programs governing the recognition of fungi (30).

These include common signaling pathways involving Syk kinase, Card9 and NF-κB downstream CLRs and ERK kinase, PI3K/Akt downstream TLRs for Th1/Th2/Th17 priming by conventional, inflammatory DCs, as well as p38/TRIF/STAT3 for Treg priming by plasmacytoid DCs (2, 31). In a mutual interaction, the host and the fungus control each other to avoid potential harmful inflammatory response. The ability of a given DC subset to respond with flexible activating programs and activation of distinct intracellular signaling pathways to the different PRR/fungal molecules' combinations confers unexpected plasticity to the DC system and pivotally contributes in shaping adaptive Th cells responses in infection and vaccination. The capacity of DCs to initiate different adaptive antifungal immune responses also depends upon specialization and cooperation between DC subsets (32). The multiple, functionally distinct, receptor/signaling pathways in DCs, ultimately affecting the local Th/Treg balance,

Table 1 | CD4$^+$ Th cell subsets in fungal infections.

Th cells		Cytokines	Functions
	Th1	IFN-γ/TNF-α	Fungal clearance Inflammation
	Th17	IL-17A/IL-17F	Defensins, neutrophil recruitment Inflammation
	Th22	IL-22	Defensins Tissue homeostasis
	Th2	IL-4/IL-13	Humoral response Allergy
	Th9	IL-9/IL-10	Tissue inflammation
	Treg	IL-10/TGF-β	Low inflammation Immunosuppression
	Tr1	IL-10	Low immunopathology

are likely successfully exploited by fungi from commensalism to infection (33).

TH1 CELLS

CD4+ Th cells exist in a variety of epigenetic states that determine their function, phenotype, and capacity for persistence, and form long-term immune memory (34). Well-balanced Th1 and Th17 cell responses are crucial in antifungal immunity and facilitate phagocytic clearance of fungal recognition, mainly through release of cytokines such as TNF-α, IFN-γ, and IL-17A and IL-17F (**Table 1**). These cytokines stimulate the disparate antifungal effector functions of phagocytes, as well as the generation of optimal T-cell-dependent immunity (2, 25). A dominant Th1 response correlates with the expression of protective immunity to fungi (2, 35) and vaccines (36, 37). Through the production of the signature cytokine IFN-γ and help for opsonizing antibodies, the activation of Th1 cells is instrumental in the optimal activation of phagocytes at sites of infection. Therefore, the failure to deliver activating signals to effector phagocytes may predispose patients to overwhelming infections, limit the therapeutic efficacy of antifungals and antibodies, and favor fungal persistency (2). Patients who are deficient in IL-12Rβ are susceptible to CMC, which is frequently recurrent or persistent (38), as well as to deep paracoccidioidomycosis (39).

TH17 CELLS

Th17 are present in the human T-cell memory repertoire to fungi (2) and inborn errors of human IL-17 immunity underlie susceptibility to CMC (40) in which both Th17 (41) and Th1 (38, 42, 43) responses are defective. Combined deficiency of the Th1 and Th17 pathway predisposes to fungal diseases (44, 45), thus emphasizing the important role played by both pathways in resistance against fungi. This could be explained with the notion that Th17 cells, although found early during the initiation of an immune response, are involved in a broad range of Th1-, Th2- and Treg-dominated immune responses (2, 46). In terms of effector functions, the ability of IL-17A to mobilize neutrophils and induce defensins may contribute to a prompt and efficient control of the infection at the different body sites. In respiratory fungal infections, Th17 cell are dispensable for resistance to the primary infection caused by *A. fumigatus* (47), but are required for vaccine-induced immunity against systemic mycoses endemic to North America (48). Thus, both Th17 and Th1 (27) cells are required for vaccine immunity to respiratory fungal pathogens.

It is intriguing that Th17 responses are down regulated by *C. albicans* (49). Regardless of the contribution of this phenomenon to infection or commensalism, this finding suggests that Th17 responses are finely tuned by fungi, as the failure to downregulate Th17 may eventually result in chronic inflammation and failure to resolve the infection (47, 50). The mechanisms that linked inflammation to chronic infection have been credited to the offending potential of IL-17A that, although promoting neutrophil recruitment, impeded the timely restriction of neutrophil inflammatory potential (51) while directly promoting fungal virulence (52). Thus, the Th17 pathway could be involved in the immunopathogenesis of chronic fungal diseases where persistent fungal antigens may maintain immunological dysreactivity. This may happen in autoimmune polyendocrine syndrome type 1 patients (APS-1) and Aire-deficient mice (53) where an excessive Th17 reactivity was observed. This finding apparently conflicts with the presence of autoantibodies against IL-22, IL-17A, and IL-17F observed in these patients (54, 55). Although correlated to susceptibility to CMC, these antibodies were also present in patients without CMC. In addition, despite the presence of antibodies to type I IFN, APS-I patients do not appear prone to recurrent viral infections. It has instead been shown that autoantibodies to pro-inflammatory cytokines may act as beneficial autoimmunity in their ability to dampen pro-inflammatory mediators and restrict self-destructive immunity (56).

TH2 CELLS

IL-4 and IL-13 act as the most potent proximal signal for commitment to Th2 reactivity that, by dampening protective Th1 responses and promoting the alternative pathway of macrophage activation, favors fungal persistence, allergy, and disease relapse. Limiting IL-4 production restores antifungal resistance (2) (**Table 1**). In atopic subjects and neonates, the suppressed DTH response to fungi is associated with elevated levels of antifungal IgE, IgA, and IgG. In CF patients, heightened Th2 reactivity associates with allergic bronchopulmonary aspergillosis and is sensitive to vitamin 3 (57). However, alternatively activated macrophages may have a protective role in defense against some respiratory fungi (58, 59) and Th2-dependent humoral immunity may afford some protection, in part by promoting Th1 immunity (60) and by altering fungal gene expression and intracellular trafficking (61–63). The efficacy of certain vaccines that elicit protective antibody strongly indicates that antibody responses can make a decisive contribution to host defense to fungi (61).

TH9 CELLS

The realization that Th effectors can produce various other cytokines alone or in combination in patterns not fitting the preconceived definitions of Th1/Th2 or Th17 subsets has led to the description of additional Th cell lineages, including Th9 and Th22. Initially thought to be a Th2-specific cytokine by virtue of its role in the pathogenesis of asthma, IgE class switch recombination, and resolution of parasitic infections, IL-9 is now considered to be the product of a distinct Th subset, the Th9 (64). Despite its relationship with other subsets, such as Th2, Th17, and Treg cells, Th9 cell subset can mediate tumor immunity and participate in autoimmune and allergic inflammation. Recently, human memory Th9 cells were found to be skin tropic or skin resident. Human Th9 cells co-expressed TNF-α and granzyme B, lacked coproduction of Th1/Th2/Th17 cytokines, and many were specific for *C. albicans*. IL-9 production preceded the upregulation of other inflammatory cytokines, such as IFN-γ, IL-13, and IL-17. IL-9-producing T-cells were increased in the skin lesions of psoriasis, suggesting that these cells may contribute to human inflammatory skin disease in the presence of *Candida* (65). Recent findings demonstrated that IL-9 is predominantly produced *in vivo* by a novel subset of innate lymphoid cells termed ILC2 (66). It has been proposed that IL-9 might have a regulatory and prosurvival function for many lymphoid and myeloid cells (67). Our recent evidence suggests that different types of ILCs are defective in IL-9-deficient mice infected with either *C. albicans* or *A. fumigatus*, and this profoundly affects the outcome of either infection and the associated pathology (unpublished observations) (**Table 1**).

TH22 CELLS

Th22 cells producing only IL-22 but neither IFN-γ nor IL-17A have been identified in human beings (68). They are induced in the presence of TNF-α and IL-6 and require ligation of aryl hydrocarbon receptor (AhR). Th22 cells via IL-22 influence the function of mesenchymal and epithelial cells and have been implicated in the dermatopathology of psoriasis and atopic dermatitis (69, 70). Memory *C. albicans*-specific IL-22+ CD4+ cells are present in human beings and defective in patients with CMC (71). Recent evidence indicates that IL-22 may play a crucial role in the innate immune resistance and local protection in mucocutaneous fungal diseases (72–74). Through the exploitation of primitive anti-fungal defense mechanisms, IL-22 was crucially involved in the control of *Candida* growth at mucosal sites in conditions of Th1 and Th17 deficiency (72, 74). Produced by ILC3 cells expressing AhR, IL-22 directly targeted gut epithelial cells to induce STAT3 phosphorylation and the release of S100A8 and S100A9 peptides known to have anti-candidal activity and anti-inflammatory effects (72, 74). Thus, due to dominant-negative mutations of STAT3, patients with autosomal dominant hyper-IgE syndrome have a defective Th17 (41) that is likely amplified on ECs where STAT3 mutation compromises the IL-22 effects. IL-22 also mediates antifungal resistance and epithelial protection in experimental and human vulvovaginal candidiasis (VVC) as well as in recurrent VVC (RVVC). In RVVC, functional genetic variants in IL22 genes were found to be associated with heightened resistance to RVVC, and they correlated with increased local expression of IL-22 (74). Thus, IL-22+ cells, employing ancient effector mechanisms of immunity, may represent a primitive mechanism of resistance against fungi under a condition of limited inflammation (**Table 1**). The fact that IL-22 production in the gut is driven by commensals (see below) also provides novel mechanistic insights on how antibiotic-related dysbiosis may predispose to candidiasis (75).

MECHANISMS OF TOLERANCE

TREG CELLS

The exposure to fungi requires the generation of a controlled immune response in the host that recognizes and controls them, limits collateral damage to self-tissues, and restores a homeostatic environment. A number of clinical observations suggest an inverse relationship between IFN-γ and IL-10 production in patients with fungal infections. High levels of IL-10, negatively affecting IFN-γ production, are detected in chronic candidal diseases, in the severe form of endemic mycoses, and in neutropenic patients with aspergillosis. Thus, high levels of IL-10 have been linked to susceptibility to fungal infections (76). However, given its prominent effect on resolution of inflammation, IL-10 production may be a consequence, rather than the cause, of the infection. This predicts that, in the case of chronic fungal infections dominated by non-resolving, persisting inflammation, IL-10 produced by Treg cells acts as homeostatic host-driven response to keep inflammation under control. Treg cells with anti-inflammatory activity have been described in fungal infections of both mice and human beings (2, 25). In experimental fungal infections, inflammatory immunity and immune tolerance in the respiratory or the gastrointestinal mucosa were all controlled by the coordinate activation of different Treg cell subsets, exerting a fine control over effector components of innate and adaptive immunity. Seen in this context, the Treg/IL-10 axis is a dangerous necessity, the failure of which may lead to detrimental inflammation. However, as the Treg responses may handicap the efficacy of protective immunity, the consequence of Treg activity is less damage to the host but also fungal persistence and immunosuppression, eventually (**Table 1**). Thus, by controlling the quality and magnitude and effector innate and adaptive responses, the spectrum of Treg cell activities may go from "protective tolerance," defined as a host's response that ensures survival of the host in a trade-off between sterilizing immunity and its negative regulation limiting pathogen elimination to overt immunosuppression. Taking a step further, this suggests that the interaction between fungi and the host immune status may determine their position from commensals to pathogens, and this position can change continuously. The salutary effects of Treg cells may go beyond their anti-inflammatory properties, to include the polarization of protective Th17 cells (46).

TR1 CELLS

T regulatory Type 1 (Tr1) cells are adaptive Treg cells characterized by the ability to secrete high levels of IL-10. Since their discovery, Tr1 cells have been proven to be important in maintaining immunological homeostasis and preventing T-cell-mediated diseases. Tr1 cells suppress T- and DC-dependent responses primarily via the secretion of IL-10 and TGF-β, release of granzyme B and perforin, and by disrupting the metabolic state of T effector cells. Tr1 cells have been demonstrated to have a role in infectious diseases, autoimmunity, and transplant rejection in different

pre-clinical disease models and in patients (77). It has recently been shown that Tr1 cells play a distinct, yet complementary role, in response to *A. fumigatus* in human beings and mice. Tr1 cells specific for an epitope derived from the cell wall glucanase Crf-1 of *A. fumigatus* (Crf-1/p41) were identified in healthy human beings and mice after vaccination with Crf-1/p41+ zymosan. These cells produced high amounts of interleukin IL-10 and suppressed the expansion of antigen-specific T-cells *in vitro* and *in vivo*, thus limiting immunopathology (**Table 1**). *In vivo* differentiation of Tr1 cells was dependent on the presence of AhR, c-Maf, and IL-27. In comparison to Tr1 cells, Foxp3+ induced Treg that recognize the same epitope were induced in an interferon gamma-type inflammatory environment and more potently suppressed innate immune cell activities. These data provide evidence that Tr1 cells are involved in the maintenance of antifungal immune homeostasis, and most likely play a distinct, yet complementary, role compared with Foxp3+ Treg cells (78).

TRYPTOPHAN METABOLISM

The enzyme indoleamine 2,3-dioxygenase 1 (IDO1) and its downstream catabolites sustain the delicate balance between Th1/Th17 pathways and Treg cells, by providing the host with adequate protective immune mechanisms without necessarily eliminating the pathogen or causing undesirable tissue damage (79). As a result of their ability to induce differentiation of Treg cells and inhibit Th17 cells, IDO1 is critical to cell lineage commitment in experimental fungal infections and contributes to the overall outcome of inflammation, allergy, and Th17-driven inflammation in these infections. Under these circumstances, the Th17 pathway, by inhibiting tryptophan catabolism, may instead favor pathology and provides evidence accommodating the apparently paradoxical association of chronic inflammation with fungal disease (19). IDO1 is a "metabolic" enzyme conserved through the past 600 million years of evolution. Initially recognized in infection because of antimicrobial activity ("tryptophan starvation" of intracellular parasites), IDO1 is now widely recognized as suppressor of acute inflammatory responses and regulator of mammalian immune homeostasis (79). Not surprising, IDO1 may represent an evasion mechanism for microbes that establish commensalism or chronic infection (79). In their capacity to induce Tregs and inhibit Th17, IDO1-expressing DCs and epithelial cells and kynurenines revealed an unexpected potential in the control of inflammation, allergy, and Th17-driven inflammation in these infections (51, 80).

MICROBIOTA REGULATION OF RESISTANCE AND TOLERANCE TO FUNGI

Commensal-driven mucosal responses are upregulated in IDO1 deficiency (81) and IL-22 responses are upregulated in conditions of defective adaptive immunity (72) and IDO deficiency (75). AhR is a ligand-activated transcription factor that mediates IL-22 production (82). A variety of indole derivatives act as endogenous ligands for AhR (83) and are generated through conversion from dietary tryptophan by commensal intestinal microbes (84). Recent evidence has shown that AhR is involved in the (patho)physiology of skin including the regulation of skin pigmentation, photocarcinogenesis, and skin inflammation (85, 86). Of interest, the ability of *Malassezia*-derived indoles to activate AhR correlated with local immunoregulation (87) and pathogenicity in seborrheic dermatitis (88). Similarly, metabolomics has revealed that bioactive indoles with Ahr agonist activity are also present in mice with candidiasis (75). Thus, the trpyptophan metabolism pathway is exploited by commensals and the mammalian host to increase fitness in response to fungi via induction of resistance and tolerance at the skin and mucosal surface. The new findings support a model in which the IL-22 axis controls the initial fungal growth (i.e., resistance) and epithelial cells homeostasis likely exploiting primitive anti-fungal effector defense mechanisms. In contrast, the exploitation of the IFN-γ/IDO 1 axis for functional specialization of antifungal regulatory mechanisms (i.e., protective tolerance) may have allowed the fungal microbiota to co-evolutes with the mammalian immune system, survives in conditions of high-threat inflammation, and prevents dysregulated immunity (79). The two pathways, although non-redundant, are reciprocally regulated and compensate each other in the relative absence of either one (72), consistent with the theme that adaptive immunity depends on innate immunity but innate immunity requires adaptive regulation. This finding not only helps to explain the association of fungal infections with dysbiosis but also points to the essential help the microbiota may provide in fungal colonization and pathogenicity in immunodeficient patients.

CONCLUSION

Vertebrates have co-evolved with microorganisms resulting in a symbiotic relationship, which plays an important role in shaping host immunity. However, intestinal inflammation also dictates the composition of gut-associated microbial communities (89), a finding indicating the reciprocal influence of the microbiota and the mammalian immune status. The mycobiome is not an exception to the rule. The activation of different Th cells with distinct effector and immunoregulatory functions may impact differently on the local mycobiome composition. Indeed, the findings that fungi oppositely react to IFN-γ (90) or IL-17A (52), in terms of growth and virulence, suggest that the local Th environment may contribute to the diversity of the mycobiome at different body sites. Ultimately, fungi have evolved a contingency-based system during co-evolution to adapt to host immunity and persist in an inflammatory host environment. In turn, this feeds back into the host immune fitness. For instance, manipulation of the regulatory network of the host by the fungal microbiota, resulting in the activation of Treg-dependent immune tolerance, is a mechanism to ensure fungal survival and commensalism at different body sites, as well as local immune tolerance (76, 91, 92). Thus, challenging existing paradigms with new perspectives from the crosstalk between fungi, the immune system, and the microbiota will eventually lead toward the development of multi-pronged therapeutic approaches for mucosal and systemic fungal diseases.

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CD4 T-cell subsets in malaria: TH1/TH2 revisited

Damian Perez-Mazliah and Jean Langhorne*

Division of Parasitology, MRC National Institute for Medical Research, London, UK

Edited by:
Dragana Jankovic, National Institutes of Health, USA

Reviewed by:
Urszula Krzych, Walter Reed Army Institute of Research, USA
Mauricio Martins Rodrigues, Federal University of São Paulo, Brazil
David K. Cole, Cardiff University, UK

***Correspondence:**
Jean Langhorne, Division of Parasitology, MRC National Institute for Medical Research, The Ridgeway, London NW7 1AA, UK
e-mail: jlangho@nimr.mrc.ac.uk

CD4$^+$ T-cells have been shown to play a central role in immune control of infection with *Plasmodium* parasites. At the erythrocytic stage of infection, IFN-γ production by CD4$^+$ T-cells and CD4$^+$ T-cell help for the B-cell response are required for control and elimination of infected red blood cells. CD4$^+$ T-cells are also important for controlling *Plasmodium* pre-erythrocytic stages through the activation of parasite-specific CD8$^+$ T-cells. However, excessive inflammatory responses triggered by the infection have been shown to drive pathology. Early classical experiments demonstrated a biphasic CD4$^+$ T-cell response against erythrocytic stages in mice, in which T helper (Th)1 and antibody-helper CD4$^+$ T-cells appear sequentially during a primary infection. While IFN-γ-producing Th1 cells do play a role in controlling acute infections, and they contribute to acute erythrocytic-stage pathology, it became apparent that a classical Th2 response producing IL-4 is not a critical feature of the CD4$^+$ T-cell response during the chronic phase of infection. Rather, effective CD4$^+$ T-cell help for B-cells, which can occur in the absence of IL-4, is required to control chronic parasitemia. IL-10, important to counterbalance inflammation and associated with protection from inflammatory-mediated severe malaria in both humans and experimental models, was originally considered be produced by CD4$^+$ Th2 cells during infection. We review the interpretations of CD4$^+$ T-cell responses during *Plasmodium* infection, proposed under the original Th1/Th2 paradigm, in light of more recent advances, including the identification of multifunctional T-cells such as Th1 cells co-expressing IFN-γ and IL-10, the identification of follicular helper T-cells (Tfh) as the predominant CD4$^+$ T helper subset for B-cells, and the recognition of inherent plasticity in the fates of different CD4$^+$ T-cells.

Keywords: malaria, *Plasmodium*, multifunctional CD4 T-cells, CD4 T-cell subsets, Tfh, Th1, Th2, Th22

INTRODUCTION

Malaria, caused by infection with *Plasmodium* transmitted via mosquito bites, represents a major global cause of morbidity and mortality (1). *Plasmodium* spp. are eukaryotic apicomplexan intracellular parasites with different life-cycle stages within the vertebrate host: an early clinically silent liver stage that can last approximately 7–10 days in humans and 2 days in rodents, followed by an erythrocytic stage, responsible for the pathology of malaria (**Figure 1A**). Species of *Plasmodium* that infect humans include *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale*, and *P. knowlesi*. A number of *Plasmodium* species that infect rodents, but not humans, are available for laboratory research, including *P. berghei*, *P. vinckei*, *P. chabaudi*, and *P. yoelii* (2), which allow the dissection of immune mechanism of protection and pathology (3).

In most cases, the host's immune system can eventually control a *Plasmodium* infection; however, exacerbated host immune responses and inflammation induced by the parasite, contribute to the pathology accompanying infection (4, 5). CD4$^+$ T-cell responses have been associated with control of erythrocytic stage parasites, but a small number of studies indicate a helper role also in pre-erythrocytic immunity (6–11). Parasite biology, host cell and tissue tropism, and kinetics of parasite growth differ between pre-erythrocytic and erythrocytic stages within the vertebrate host and, accordingly, the particular CD4$^+$ T-cell responses elicited also differ. Herein, we discuss activation of different CD4$^+$ T-cell subsets during malaria, their role in the control of the infection and the interplay between different subsets, with a particular emphasis on the concept of CD4$^+$ T-cell plasticity.

CD4$^+$ T-CELL SUBSETS ACTIVATED BY PRE-ERYTHROCYTIC STAGES

Very little is known about the CD4$^+$ T-cell response to *Plasmodium* pre-erythrocytic stages, or its regulation in natural infection either in humans or in experimental models. Clearly, since IgG antibodies and memory B-cells are generated to a wide range of pre-erythrocytic antigens, including those with expression restricted primarily to these stages, such as circumsporozoite protein (CSP), liver-stage antigen 1 (LSA1), and sporozoite threonine–asparagine-rich protein (STARP) (12–14), CD4$^+$ T-cells must be induced by these stages of the infection. Indeed, CD4$^+$ T-cells specific for pre-erythrocytic antigens have been documented, and in some cases, have been shown to correlate with protection in humans following natural infection (15) and immunization (11). However, we have few details of their functional heterogeneity.

CD4$^+$ T-cells of undefined Th1/Th2 phenotype have been shown to confer protection against the pre-erythrocytic stages of *P. yoelii* even in the absence of CD8$^+$ T-cells (9), and CD4$^+$ T-cell clones recognizing peptides of CSP protected against a *P. yoelii* sporozoite challenge in mice, irrespective of their Th1 or

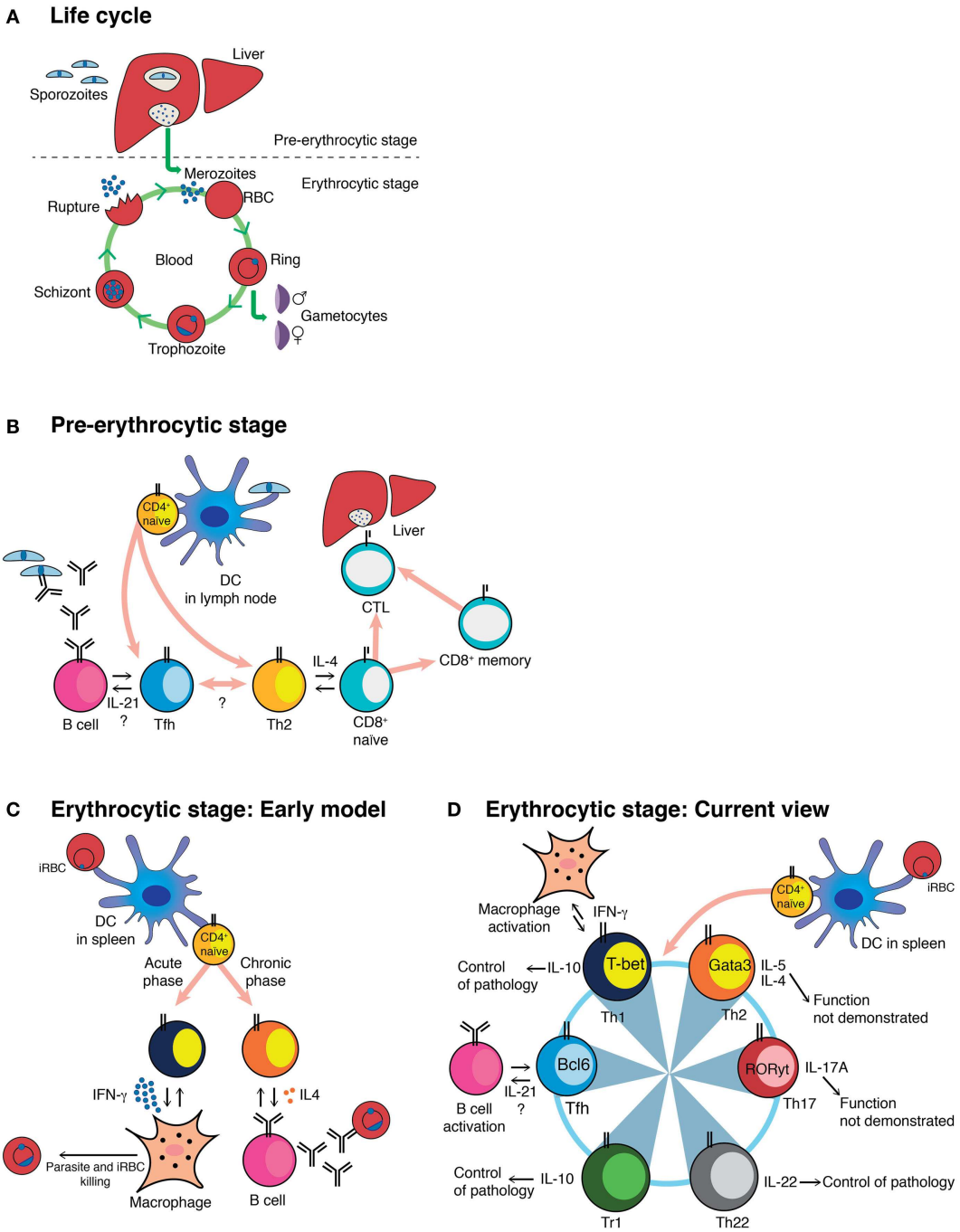

FIGURE 1 | Schematic representation of the *Plasmodium* life cycle, and different models of CD4$^+$ T-cell activation during *Plasmodium* infection. **(A)** *Plasmodium* life cycle in the mammalian host. **(B)** The cartoon shows the different subsets known, or proposed to be, activated by the pre-erythrocytic stage of *Plasmodium*, together with their known or proposed functions. **(C)** Classical view of the biphasic activation of Th1 and Th2 CD4$^+$ T-cells toward the erythrocytic stage of *Plasmodium*. **(D)** Current understanding of the CD4$^+$ T-cell responses to the erythrocytic stage of *Plasmodium*, together with their known or proposed roles during infection. This model highlights the plastic ability of activated CD4$^+$ T-cells to interconvert into different Th subsets. The master regulator transcription factors known to drive each Th program as well as the cytokines associated to each Th subset are depicted (53). DC, myeloid dendritic cell; iRBC, infected red blood cell; CTL, cytotoxic CD8$^+$ T-cell; Tr1, Foxp3$^-$ regulatory T-cell.

Th2 phenotype (6, 7). The location of priming of CD4$^+$ T-cell specific for pre-erythrocytic stages is still a matter of debate, and there is little evidence as yet on priming of CD4$^+$ T-cell in the liver. As protective CD8$^+$ T-cells specific for a peptide of CSP from *P. yoelii* can be primed by dendritic cells (DCs) in lymph nodes after infection with sporozoites (16), it is likely that DCs

in lymph nodes might also be critical for priming $CD4^+$ T-cell responses to *Plasmodium* pre-erythrocytic stages.

As well as possible direct killing of infected hepatocytes, subsets of $CD4^+$ T-cells provide crucial help for both B-cell and $CD8^+$ T-cell responses (**Figure 1B**). In a very few studies, $CD4^+$ T-cells were shown to be necessary to ensure survival of protective effector and memory $CD8^+$ T-cells induced by irradiation-attenuated sporozoites (8, 10). This mechanism is dependent on STAT-6 and IL-4, suggesting that Th2 $CD4^+$ T-cells may be in charge of providing help to $CD8^+$ T-cells. More recently, CSP-specific $CD4^+$ T-cells expressing CD107a (LAMP-1), a marker for cytotoxic degranulation, were shown to be induced and associated with protection against the pre-erythrocytic stages after immunizations of healthy volunteers by bites from *P. falciparum*-infected mosquitoes during chloroquine chemoprophylaxis (11). Nothing is yet known about induction of Tfh cells, the subset that provides help for T-cell dependent B-cell responses (17), which are presumably activated by, and would be important for, generation of high affinity IgG antibodies.

Altogether, these data suggest a role of $CD4^+$ T-cells in protective immunity to pre-erythrocytic-stage infection, the mechanisms of which are not yet completely understood. With the current emphasis on pre-erythrocytic vaccines, it is important that we understand more about the potential contribution of diverse $CD4^+$ T-cell populations on direct killing of infected cells, in the B-cell and $CD8^+$ T-cell responses, as well as their regulatory roles in immunity to the pre-erythrocytic stages of *Plasmodium*.

$CD4^+$ T-CELL SUBSETS ACTIVATED BY ERYTHROCYTIC STAGES

The Th1/Th2 paradigm proposed by Mosmann and Coffman postulated stable lineages of activated $CD4^+$ T-cells with distinctive cytokine production patterns and functional capacity; IFN-γ-producing Th1 cells being crucial mediators of host immunity against intracellular pathogens, while IL-4-producing Th2 cells mediating immunity toward extracellular pathogens and collaboration with B-cells for antibody production (18). As *Plasmodium* invades red blood cells (**Figure 1A**), which do not express MHC class I or II, it was difficult to envisage parasites at this stage as direct targets of Th1 or Th2 cells. Nonetheless, it was possible to draw parallels with the original Mosmann and Coffman model; malaria researchers observed that the erythrocytic stages triggered a strong IFN-γ response during acute infections in *P. berghei*, *P. yoelii*, and *P. chabaudi* infections in mice, as well as in *P. falciparum* infection in humans (19–24).

Since that time, the Th1/Th2 paradigm has gained in complexity with the identification of novel $CD4^+$ T-cell subsets with distinctive characteristics and transcriptional programs in charge of driving the different cell fates (25). These differentiation programs are governed predominantly by signals derived from antigen-presenting cells (APC) and the microenvironment at the time of $CD4^+$ T-cell activation. DCs are necessary for effective priming of the T-cell response in erythrocytic-stage malaria (26), and two subsets of splenic DCs, $CD8^-$ and $CD4^+$ classical DCs, have been shown to present antigen for the activation of $CD4^+$ T-cells during an erythrocytic-stage infection with *P. chabaudi* and *P. berghei*, respectively (26–29). Although it is known that IL-12 is an important cytokine in the induction of a protective response in experimental malaria infections (30), understanding of the regulation of this cytokine or other factors in DCs necessary for effective *Plasmodium*-antigen presentation to different subsets of $CD4^+$ T-cells is still lacking.

IFN-γ, a defining cytokine of Th1 cells expressing the transcription factor T-bet, has proven to be important for controlling the acute erythrocytic stage of *Plasmodium* infection in rodent models (31–34). IFN-γ-producing $CD4^+$ effector (E) and effector memory (EM) $CD4^+$ T-cells both confer partial protection from *P. chabaudi* infection (35). In general agreement with this, IFN-γ from $CD4^+$ T-cells has been shown to be important in maintaining strain-transcending blood-stage immunity (36). However, IFN-γ is not only produced by T-bet^+ Th1 cells but also by NK cells, NKT cells, and γδ T-cells (37, 38) as well as $CD8^+$ T-cells, and it is not always clear whether Th1 cells, IFN-γ, or IFN-γ from Th1 cells *per se* are the main players in early protection or pathology in experimental malaria. Studies so far to address these questions have given conflicting results. One study using *P. berghei* ANKA has shown that the enhanced IFNγ^+ T-bet^+ $CD4^+$ T-cell responses observed in mice lacking Type I IFN signaling are associated with better control of *P. berghei* ANKA infections, resulting in lower morbidity and mortality (39, 40). In contrast, others have shown that in the absence of T-bet, essential for Th1 commitment, cerebral pathology of *P. berghei* ANKA infections is ameliorated, and the number of IFNγ^+ $CD4^+$ T-cells is reduced. However, control of parasite replication is lost and mice succumb to hyper-parasitemia and anemia (41). In a different rodent model of erythrocytic-stage malaria, *P. yoelii* 17X(NL), although activation of T-bet was detected on $CD4^+$ T-cells early in infection (42), the infection can still be controlled in T-bet-deficient mice (43).

The erythrocytic stages of *Plasmodium* are also able to activate $CD4^+$ T-cells that are very effective helpers for *Plasmodium*-specific antibody production, but produce little or no IFN-γ (21). This response was shown to coincide with the appearance of IL-4-producing $CD4^+$ T-cells (21). The association between IL-4-producing $CD4^+$ T-cells and antibody responses toward the parasite was also observed in *P. falciparum* immune subjects (44). An erythrocytic-stage *P. chabaudi* infection, the only mouse model that generates a chronic phase of infection (3), presents a biphasic $CD4^+$ T-cell activation, with a large IFN-γ-producing $CD4^+$ T-cell response during the acute phase, followed by an antibody-helper/IL-4-producing $CD4^+$ T-cell response during the chronic phase (**Figure 1C**) (21, 24, 45, 46). These data were interpreted as an early activation of Th1 cells able to control parasitemia through the activation of effector mechanisms such as macrophages, followed by a Th2 response in charge of activating B-cell responses to complete the clearance of the parasite (47, 48). However, the frequency of $CD4^+$ T-cells able to help B-cells to produce *Plasmodium*-specific antibodies was much higher than the frequency of IL-4-producing $CD4^+$ T-cells (21). Furthermore, control of a *P. chabaudi* infection and specific IgG responses, including IgG1 antibodies, was possible even in the complete absence of IL-4 (45). Therefore, it was clear, despite its attractiveness as a model, that the simple Th1/Th2 paradigm was not sufficient to explain the full complexity of $CD4^+$ T-cell activation in the erythrocytic stages of *Plasmodium*. More recently, a subset of $CD4^+$ T-cells, Tfh cells,

has been described that produce IL-21, as well as other cytokines originally associated with other Th subsets, such as IFN-γ and IL-4 (17). We believe that the Tfh program, and not a Th2 response, is the critical one for B-cell help and activation of protective B-cell responses against the erythrocytic stages of *Plasmodium* infection (**Figure 1D**). However, there are very few data on Tfh or its crucial signature cytokine, IL-21, in malaria. Lymphocytes closely resembling Tfh have been observed in peripheral blood of humans (49), although not yet in people exposed to malaria. However, IL-21-producing $CD4^+$ T-cells have been demonstrated in blood of immune adults living in endemic areas of *P. falciparum* transmission (50–52). Given the importance of the humoral response in protective immunity to the erythrocytic stages of *Plasmodium*, understanding the activation and maintenance of Tfh cells during malaria is of outstanding interest for vaccine design.

Recently, the Th17 subset of $CD4^+$ T-cells, defined by the expression of the transcription factor RORγt, has gained attention among malaria researchers because of its role in autoimmune diseases and chronic inflammation and in responses to extracellular pathogens such as bacteria and fungi (53). $CD4^+$ IL-17A$^+$ RORγt$^+$ Th17 cells are activated during acute *P. berghei ANKA* and *P. yoelii* infection, but the function of these cells during infection was not explored (54). Ishida and colleagues demonstrated no association of Th17 cells and cerebral malaria in *P. berghei ANKA*-infected IL-17-deficient mice (55). We have also observed the presence of IL-17A and IL-17F-producing $CD4^+$ T-cells mainly in the liver during acute erythrocytic-stage *P. chabaudi* infection; however, IL-17A-deficient mice showed no significant alterations in the course of *P. chabaudi* infection (56). Therefore, despite activation, Th17 cells have so far not been shown to have a defined role during *Plasmodium* infections (**Figure 1D**).

Additional $CD4^+$ T-cell subsets, such as that producing IL-22 (Th22), continue to be identified (57, 58). IL-22 has been implicated in both host defense against bacterial infections and tissue repair (59). Interestingly, IL-22 single-nucleotide polymorphisms associated with resistance and susceptibility to severe malaria have been identified (60). We have observed that IL-22-producing $CD4^+$ T-cells are activated, albeit in low frequency, in both spleen and liver during acute erythrocytic-stage *P. chabaudi* infections, and that IL-22-deficient mice infected with *P. chabaudi* show exacerbated pathology (56) (**Figure 1D**). Research is ongoing to explore in greater detail the origins of these cells, their location, and the mechanisms underlying the observed pathology.

With the discovery of this wide array of possible $CD4^+$ T-cell subsets and their different activation requirements and functional capacities, it is becoming clear that $CD4^+$ T-cells may not be simply defined as individual subsets of Th cells producing a single cytokine, but rather they represent components of a dynamic and interactive response, in which these cells can be multifunctional, flexible, and plastic depending on the disease/infection and activation environment (61). The multifunctional capacity of T-cells, or the ability to perform more than one function (e.g., production of different cytokines) at the single-cell level, and its association with the capacity to control infections was first recognized in HIV-infected subjects (62) and in a mouse model of vaccination against *Leishmania major* (63). This association between multifunctional capacity of T-cells and control of infections is not limited to HIV and *Leishmania*, and was soon observed in several other chronic infections, including viral, parasitic, and mycobacterial infections (64). Immunization of subjects with *P. falciparum* apical membrane antigen 1 (AMA1) (65), and immunizations of mice with full-length *P. falciparum* CSP protein (66) also activates multifunctional $CD4^+$ T-cells responses. Moreover, multi-parameter flow cytometric analyses of human PBMC from children and adults exposed to malaria infection reveal the existence of $CD4^+$ T-cells co-expressing several cytokines characteristic of many $CD4^+$ T-cell subsets (52, 67), demonstrating the complexity of the $CD4^+$ T-cell response activated by the erythrocytic stages of *Plasmodium*.

There is an important body of evidence suggesting that, far from being terminally differentiated stable lineages, the different Th subsets have an extensive capacity to interconvert further between different phenotypes, a concept known as plasticity (68). The most recent studies suggest that $CD4^+$ T-cell activation with overlapping characteristics of different Th subsets is the norm rather than the exception (61), and this is likely to be reflected in complex diseases such as malaria. Although this has not been explored in any detail in the context of malaria, the concept of Th plasticity opens new possibilities for studying the function and regulation of $CD4^+$ T-cells in the control of *Plasmodium* infection and related immunopathology. One subset known for its remarkable plasticity is the Tfh subset. It has been shown that Th1, Th2, and Th17 cells can migrate into the B-cell areas of secondary lymphoid organs and acquire the functional capacity and biomarkers of Tfh cells and, conversely, the Tfh subset can become Th1, Th2, and Th17 (69). In the context of *Plasmodium* infection, this would imply that not only could *Plasmodium* parasites activate Tfh responses directly but also the Tfh subset could potentially arise from any of the $CD4^+$ T-cell subsets already activated by the erythrocytic stages.

In light of the multifunctional and plastic capacities of the $CD4^+$ T-cells, a scenario can be envisaged in which Th subsets required for the control of parasite burden, such as Th1 cells, have the capacity to acquire a regulatory phenotype depending on the context, contributing to control of the inflammation and thus to protection of tissues and organs and preventing any potentially harmful effects of the response. This would allow a fine-tuning of the $CD4^+$ T-cell response to guarantee the control of *Plasmodium* infection without causing deleterious side effects. One mechanism of self-regulation by $CD4^+$ Th1 cells in malaria is the induction of IL-10. IFN-γ$^+$IL-10$^+$T-bet$^+$ Th1 $CD4^+$ T-cells can prevent pathology during *P. chabaudi* infection (70) (**Figure 1D**). In addition, IL-10 from $CD4^+$ T-cells distinct from regulatory T (Treg) cells is able to control pathology in a *P. yoelii* infection in mice, but in this case, these cells do not co-express IFN-γ (71). IL-10 is produced in these cells in response to IL-27 (70), although the signals responsible for the induction of IL-27 remain unknown. The occurrence of IFN-γ$^+$IL-10$^+$T-bet$^+$ $CD4^+$ T-cells during *Plasmodium* infections is not restricted to mouse models; they have been reported to be present in PBMC of children living in highly malaria-endemic regions (72–74) and their proportion is higher in children with uncomplicated malaria compared to children with severe malaria (72). IL-10 can also be induced in IL-17-producing $CD4^+$ T-cells, as yet by unknown pathways (75–77), and thus, IL-10 may be a more general mechanism for regulating any subset

of $CD4^+$ T-cells in malaria. $CD4^+$ T-cells, particularly Th1 cells, can also be controlled by Type I IFNs. In the *P. berghei* ANKA model, Type I IFN signaling suppresses Th1 responses by directly acting on classical DCs (40). Given that type I IFN signaling can also promote the expression of IL-10 on $CD4^+$ T-cells (78–81), we hypothesize that these two regulatory mechanisms might share some common activation signals during *Plasmodium* infection.

$CD4^+$ T-cell responses may also be controlled by the expression of surface molecules associated with exhaustion. Elevated frequencies of PD-1^+ LAG-3^+ $CD4^+$ T-cells have been reported in *P. falciparum*-infected subjects (82, 83), and combined blockade of PD-1 and LAG-3 accelerated clearance of erythrocytic-stage *Plasmodium* infection in a mouse model (83). In agreement with these observations, PD-1-deficient mice show better control of an erythrocytic-stage *P. chabaudi* infection with higher frequencies of IFN-γ^+ and T-bet$^+$ $CD4^+$ T-cells during the chronic phase (84). The kinetics of PD-1^+ $CD4^+$ T-cells during the acute erythrocytic-stage *P. yoelii* 17X(NL) infection are similar to those observed during *P. chabaudi* infection (42, 84). However, some caution should be exercised in assuming that expression of PD-1 automatically means exhaustion, as in some subsets of activated $CD4^+$ T-cells, in particular Tfh cells, PD1 is expressed without affecting their functional capacity. It may be that the triggering of PD-1 by its ligand PDL-1 (85) is the key to whether the cell is programed for cell death.

Many of the $CD4^+$ T-cells activated in a *Plasmodium* infection may undergo interconversion between defined cell subsets depending on antigen dose, APC, location, and cytokine/chemokine environment, such as that described for Treg and Th17 subsets (86, 87). Thus, it is possible that the Th17 cells found in the spleen of malaria-infected mice gain a regulatory phenotype in other organs or tissues such as brain and liver. The capacity to identify and manipulate these possible mechanisms of $CD4^+$ T-cell plasticity during *Plasmodium* infections would be of great value for the design of novel therapeutic strategies.

CONCLUDING REMARKS

The identification of two $CD4^+$ T-cell subsets with different well-defined functions represented an attractive organizational system with which to rationalize $CD4^+$ T-cell responses to *Plasmodium* infections. However, such a model has not been sufficient to reflect fully the complexity of $CD4^+$ T-cell biology observed in human or experimental malaria. In particular, control of *Plasmodium* infection requires strictly regulated immune responses that are able to prevent parasite replication without causing detrimental side effects of uncontrolled inflammation. *Plasmodium* species have different stages with different tissue tropisms and this complex life cycle challenges the idea that a single static group of terminally differentiated $CD4^+$ T-cells would be able to perform all the tasks required to control this infection. In order to cope with these tasks, the $CD4^+$ T-cell response has to adapt to the changing scenarios presented as the infection evolves. The newer concept of $CD4^+$ T-cell plasticity would add substantially to our understanding of induction and regulation of $CD4^+$ T-cell responses in malaria, and it is highly probable that some of the $CD4^+$ Th programs not yet explored in depth, such as the Tfh response, might play critical roles in the outcome of the infection. The combination of potent tools such as multi-parameter flow cytometry, *in vivo* imaging, systems analyses of transcriptome, proteome, and metabolome, together with T-cell receptor transgenic mice and peptide-MHC II tetramers will give us the chance to explore the complexity of the $CD4^+$ T-cell responses to malaria in *in vivo* models (74, 88–97). In addition, confocal microscopy and intravital imaging techniques make now possible to follow sporozoites injected via the mosquito bite into the skin, or by injection of attenuated sporozoites through to their arrival in the lymphoid organs and liver (98), and to study the consequent activation of $CD4^+$ T-cells and their subsequent effector functions. Field studies of natural human *Plasmodium* infections and mouse models should complement each other to get a deeper understanding of the complex $CD4^+$ T-cell response activated by these infections. A detailed delineation on how $CD4^+$ T-cells modulate the activation of effector cells such as $CD8^+$ T-cells, macrophages, and B-cells in response to *Plasmodium* infection is critical to achieve the goal of generating protective treatments to control malaria.

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The regulation of CD4$^+$ T cell responses during protozoan infections

Christian R. Engwerda[1]*, Susanna S. Ng[1,2] and Patrick T. Bunn[1,3]

[1] *QIMR Berghofer Medical Research Institute, Brisbane, QLD, Australia*
[2] *School of Natural Sciences, Griffith University, Nathan, QLD, Australia*
[3] *Institute of Glycomics, Griffith University, Gold Coast, QLD, Australia*

Edited by:
Dragana Jankovic, National Institutes of Health, USA

Reviewed by:
Sid P. Kerkar, National Institutes of Health, USA
Kevin Couper, University of Manchester, UK

****Correspondence:***
Christian R. Engwerda, QIMR Berghofer Medical Research Institute, 300 Herston Road, Herston, QLD 4005, Australia
e-mail: chrise@qimr.edu.au

CD4$^+$ T cells are critical for defense against protozoan parasites. Intracellular protozoan parasite infections generally require the development of a Th1 cell response, characterized by the production of IFNγ and TNF that are critical for the generation of microbicidal molecules by phagocytes, as well as the expression of cytokines and cell surface molecules needed to generate cytolytic CD8$^+$ T cells that can recognize and kill infected host cells. Over the past 25 years, much has been learnt about the molecular and cellular components necessary for the generation of Th1 cell responses, and it has become clear that these responses need to be tightly controlled to prevent disease. However, our understanding of the immunoregulatory mechanisms activated during infection is still not complete. Furthermore, it is apparent that although these mechanisms are critical to prevent inflammation, they can also promote parasite persistence and development of disease. Here, we review how CD4$^+$ T cells are controlled during protozoan infections and how these regulatory mechanisms can influence parasite growth and disease outcome.

Keywords: protozoan parasites, Th1 cells, IL-10, IL-27, CD4$^+$ T cells, immune regulation

INTRODUCTION

Mammalian immune systems have evolved to recognize and control pathogens. This is achieved by the coordinated actions of innate and adaptive immune mechanisms [reviewed in Ref. (1, 2)]. CD4$^+$ T cells play key roles in coordinating immune responses by producing molecules critical for the production of high affinity antibodies by B cells and promoting the production of mucous and tissue repair mechanisms. They also help to fully activate CD8$^+$ T cells so they can kill infected and transformed cells, and assist innate immune cells to recognize and control pathogens and tumors. CD4$^+$ T cells play critical roles in both the generation of anti-parasitic immunity and immune surveillance during concomitant immunity, which is associated with many parasitic infections (3).

REGULATION OF T CELL RESPONSES

The help provided by CD4$^+$ T cells for various immune activities includes the production of potent pro-inflammatory cytokines such as TNF, IFNγ, and IL-17, and as such, CD4$^+$ T cell responses need to be tightly regulated so they themselves do not cause tissue damage. The pathogenesis of autoimmune diseases often involves aberrant CD4$^+$ T cell responses in tissue sites such as the central nervous system, pancreas, and brain. Therefore, mammals have evolved multiple ways to control the pathogenic potential of CD4$^+$ T cells [reviewed in Ref. (4)]. These include indoleamine 2,3-dioxygenase (IDO)-catalyzed tryptophan metabolism by phagocytic cells (5), leading to immune cell stress and activation of the general controlled non-repressed 2 (GCN2) kinase pathway (6) and/or cytotoxic and regulatory effects on T cells caused by the catabolites from the associated kynurenine metabolism pathway (7). In addition, the production of regulatory cytokines, such as IL-10 and TGFβ, by innate immune cells in response to pathogen-derived molecules can suppress both developing and established T cell responses (8–10), as can IL-10 produced by certain B cell subsets (11). Dendritic cells (DCs) can be an important source of regulatory cytokines in experimental models of leishmaniasis and malaria. In addition, over the course of these infections, DCs reduce levels of CD11c, increase expression of CD45RB, and promote the generation of T cell IL-10 production (12–14). Thus, the development of regulatory DC subsets that have a major influence on T cell responses is a feature of established protozoan infections. More recently, specialized monocytes and macrophage subsets have been identified that can modulate localized T cell responses during protozoan infections [reviewed in Ref. (15)]. Classically activated (M1) macrophages produce pro-inflammatory molecules, such as TNF and L-arginine-dependent nitric oxide, while alternatively activated (M2) macrophages use arginase 1 to convert L-arginine to polyamines, which along with production of IL-10 and TGFβ, enable this cell subset to suppress inflammation [reviewed in Ref. (16)]. Inflammatory monocytes have been reported to promote Th1 cell activity in mice infected with *Leishmania major* (17), *L. donovani* (18), and *Trypanasoma brucei* (19, 20), but with pathological consequences in the latter model that were reversed by administration of IL-10 (20). In contrast, the products from M2 macrophages suppressed lesional CD4$^+$ T cell proliferation and IFNγ production in mice infected with *L. major* (21), while *T. gondii* can actively promote the arginase 1 pathway in macrophages to enhance pathogen survival (22). Thus, macrophages play important roles in conditioning local tissue

environments and determining the direction and effectiveness of T cell responses during protozoan infections. However, regulatory mechanisms increasingly recognized as being paramount for preventing T cell-mediated disease, and therefore, the main subject of this review, involve specialized sub-populations of $CD4^+$ T cells themselves capable of inhibiting immune responses and suppressing inflammation.

REGULATORY T CELLS

Regulatory T cells can be broadly divided into two types. First, natural regulatory T (Treg) cells are $CD4^+$ T cells produced in the thymus and express the transcription factor FoxP3 that is critical for their suppressive functions (23, 24). Second, inducible regulatory T cells emerge from the thymus as conventional T cells, but develop regulatory functions in the periphery following exposure to appropriate inflammatory stimulation. These include IL-10-producing Th1 (Tr1) cells (25), TGFβ-producing $CD4^+$ T (Th3) cells (26), and conventional $CD4^+$ T cells that have converted to FoxP3-positive cells in peripheral tissues (27). Under homeostatic conditions, Treg cells limit potentially self-reactive T cell responses, thus preventing autoimmunity (23). However, they can also impair effective pathogen clearance, while trying to prevent immune-mediated tissue damage during infection. The molecular mechanisms by which Treg cells perform these functions are incompletely understood, but involve production of cytokines such as IL-10, TGFβ, and IL-35, the expression of the negative regulatory molecule cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) and the generation of adenosine and cyclic AMP [reviewed in Ref. (28)]. In addition, their expression of high affinity IL-2 receptor allows them to deprive conventional T cells of this critical growth factor and thereby induce them to undergo apoptosis (29). Thus, Treg cells may act directly upon conventional T cells or via accessory cells such as antigen presenting cells (APCs) to limit T cell activity. An emerging paradigm is that Treg cells adapt to particular inflammatory conditions in order to regulate specific $CD4^+$ T cell responses by the generation and use of shared transcription factors to mimic certain aspects of T cell behavior, such as tissue homing, survival, and cytokine production. For example, STAT3, T-bet, IRF-4, and Bcl-6 are required for Th17, Th1, Th2, and follicular helper T (Tfh) cell differentiation, respectively, as well as by the Treg cells that control the actions of these specific $CD4^+$ T cell subsets (30–34). For example, in mice orally infected with *T. gondii*, IL-27 promotes the expression of CXCR3 on Treg cells that enables these cells to regulate Th1 cell-mediated immunity, as well as prevent infection-induced pathology at mucosal sites (35). However, Treg cells can block the generation of effective parasite-specific T cell responses in specific tissues. For example, Treg cell depletion with anti-CD25 mAb in mice infected with *L. major* dramatically enhanced anti-parasitic immunity (36), while adoptive transfer of antigen-specific Treg cells in the same model promoted parasite growth (37). In addition, depletion of Treg cells with an anti-CD25 mAb protects mice from lethal *Plasmodium yoelii* infection by enabling the generation of a potent anti-parasitic T cell response (38). Similarly, the removal of Treg cells from peripheral blood mononuclear cells isolated from humans infected with *P. falciparum* enhanced T cell proliferation and $CD4^+$ T cell IFNγ production in response to stimulation with parasite antigens (39). However, the importance of Treg cells in several protozoan infections has been questioned because of the potential "off-target" effects of the anti-CD25 mAbs used in many studies (40). For example, several groups reported significant changes in immune responses and disease outcome in mice infected with *P. berghei* ANKA (41–43), but subsequent experiments in this model, where Treg cells could be specifically depleted with diphtheria toxin via cell-specific expression of a simian diphtheria toxin receptor (44), showed little impact of Treg cells on disease outcome and associated T cell responses (45, 46). Thus, the roles of Treg cells in protozoan infections will require further studies before their impact on anti-parasitic immune responses can be fully appreciated.

The secretion of IL-10 by conventional $CD4^+$ T cells can potently suppress inflammation and tissue damage (47, 48). Initially, IL-10 production was identified in Th2 cells (49), but has since been described in Th1 (50–52) and Th17 (53) cell populations. Thus, $CD4^+$ T cell-derived IL-10 production is emerging as an important mechanism of auto-regulation, whereby IL-10 can both directly suppress T cell activities, as well as upstream activation pathways initiated by APCs [reviewed in Ref. (48)]. These IL-10-producing Th1 cells were identified in mice infected with *T. gondii* (54) and *L. major* (55). In the *T. gondii* infection model, these cells did not impact upon control of parasite growth, but were critical for limiting pathology (54, 56), while in mice infected with *L. major*, IL-10-producing Th1 cells promoted the establishment and maintenance of chronic infection (55). Similar observations have also been made in mouse models of *Plasmodium* infection (57, 58), *T. cruzi* (59, 60), and *T. brucei* (61) infections. Importantly, these IL-10-producing Th1 cells have been identified in humans with visceral leishmaniasis caused by *L. donovani* (62) and African children with *P. falciparum* malaria (63, 64). Although IL-10 has been clearly shown to suppress $CD4^+$ T cell activation in humans infected with *L. donovani* (65) and *P. falciparum* (63, 66), it is not yet clear how much of this activity can be attributed to the IL-10-producing Th1 cells. Significantly, the prevalence of IL-10-producing Th1 cells in Gambian children with asymptomatic malaria was greater than in children with severe disease, indicating that these cells may protect against damaging inflammation during acute malaria (67). However, antigen-specific IL-10-producing Th1 cells were found in cord blood of babies whose mothers had malaria during pregnancy (66), suggesting that these cells might be able to influence anti-parasitic immunity from very early in life. Hence, the kinetics of the emergence of IL-10-producing Th1 cells during malaria may be critical in determining the impact they have on the outcome of infection.

THE ROLES OF IL-10 IN PROTOZOAN INFECTIONS

IL-10 is one of the most potent regulatory cytokines produced by leukocytes in response to inflammatory signals (68). The importance of IL-10 for regulating immunity is highlighted by the observation that IL-10 deficiency or blockade causes the early development of colitis in mice (69). However, as described above, many protozoan parasites, such as those that cause toxoplasmosis, malaria, trypanosomiasis, and leishmaniasis (54, 55, 59–61,

70, 71), have evolved to exploit the functions of IL-10 to inhibit anti-microbial mechanisms and allow the establishment of chronic infection. In fact, the generation of IL-10-producing T cells following vaccination with protozoan antigen can be a robust predictor of vaccine failure (28). One proposed mechanisms for IL-10-mediated immune suppression is the promotion of T cell exhaustion. The PD-1 pathway plays an important role in T cell exhaustion during all of the chronic infections mentioned above, and there is strong evidence that IL-10 plays a key role in regulating the expression of the PD-1 ligands (PD-L1 and PD-L2) on APCs [reviewed in Ref. (72)]. Several other molecules known to be involved in promoting T cell exhaustion, such as Tim-3 and Lag3, have also been linked with IL-10 expression (73, 74), but their precise relationships are not known. IL-10 produced by macrophages can also inhibit the differentiation of surrounding cells into classically activated macrophages that are required for the production of inflammatory cytokines and metabolites required to kill many intracellular pathogens (75). It can also suppress inflammatory cytokine production by T cells and inhibits antigen presentation by APC [reviewed in Ref. (76)]. Thus, IL-10 can suppress host immune responses during infection by multiple mechanisms.

REGULATION OF IL-10 PRODUCTION BY CD4$^+$ T CELLS

An important approach to understanding how IL-10 production might be modulated for therapeutic advantage or to improve vaccination is to gain a better insight into the transcriptional regulation and the signaling pathways involved in IL-10 production and establishing whether they differ between cell types and in various tissue locations. IL-27 has emerged as an important growth and differentiation factor for IL-10-producing Th1 cells (53, 77, 78). It is thought to primarily be a product of macrophages and DCs (79), and drives the production of IL-21 by CD4$^+$ T cells, which in turn, acts as an autocrine growth factor for IL-10-producing Th1 cells (80, 81). IL-27 is a heterodimeric cytokine composed of IL-27p28 and EBI3 that signals via a receptor complex comprising a unique IL-27 receptor alpha chain (IL-27Rα) and gp130 (82, 83), a common receptor used by several cytokines including IL-6 (84, 85). IL-27 promotes these activities via the transcription factors STAT1 and STAT3 (53), and by inducing the expression of the transcription factors c-Maf (80) and aryl hydrocarbon receptor (AhR) (86), which then physically associate and transactivate the IL-10 and IL-21 gene promoters (80, 86, 87). Interactions between glucocorticoid-induced TNFR-related (GITR) protein and GITR ligand can also stimulate IL-27 production (88), which can induce expression of inducible T cell costimulator (ICOS) on IL-10-producing Th1 cells to enhance IL-27-mediated expansion of these cells (80). Interestingly, IL-27p28 can also function as a natural antagonist of gp130-mediated cytokine signaling, and thereby inhibit IL-6-mediated inflammatory pathways (89). The importance of IL-27 for the generation of IL-10-producing Th1 cells has now been reported in mouse models of malaria (58, 90), leishamaniasis (91), and toxoplasma (53), although, surprisingly, the generation of these cells was independent of IL-21 in mice infected with *P. chabaudi* (58). It should also be noted that IL-27 has IL-10-independent regulatory functions in mice infected with *P. berghei* NK65 (90), thus emphasizing the complexity of IL-27-mediated immune regulation during protozoan infections. IL-27 produced by CD14 positive monocytes was also reported to be associated increased numbers of IL-10-producing Th1 cells in blood from visceral leishmaniasis patients (92). Thus, there is substantial evidence for IL-27 being a critical factor in the generation of IL-10-producing Th1 cells during protozoan infections.

In other studies, the transcriptional repressor B lymphocyte-induced maturation protein 1 (Blimp1; encoded by the *Prdm1* gene) was found to be expressed by a subset of Treg cells and played an essential role in their production of IL-10 (93). Recently, Blimp1 was implicated in IL-10 production by Th1 cells (94), and shown to be important for the generation of these cells in mice infected with *T. gondii* (95). IL-27 and T cell receptor signaling were found to promote the expression of the transcription factor Erg2, which was required for Lag3 expression and production of IL-10 by conventional CD4$^+$ T cells (96). Subsequently, IL-27-dependent Egr2 expression was reported to be critical for the induction of Blimp1 and generation of IL-10-producing Th1 cells (94). Interestingly, only STAT3-deficiency impacted upon IL-27-dependent Egr2 expression, while both STAT1 and STAT3 were required for IL-10 production by Th1 cells. Thus, a model for IL-10 production by Th1 cells is emerging (**Figure 1**). Furthermore, cellular pathways such as the Notch-Jagged axis in plasmacytoid DCs promote CD4$^+$ T cell IL-10 production (97), but their roles in protozoan infections have yet to be investigated. Thus, there are still many gaps to be filled, and importantly, we need to clearly define the signaling and transcriptional pathways that are activated during protozoan infections. In particular, there is a clear gap in our knowledge regarding differences in the regulation and maintenance of IL-10 production by Th1 cells in secondary lymphoid organs and peripheral tissue sites. This information is important if we want to target these regulators to selectively modulate IL-10 activity during parasitic disease. In the broader context of immune regulation, we need to establish whether the IL-10-producing Th1 cells are a distinct T cell subset capable of dynamic and sustained regulatory function or whether they represent exhausted T cells, as suggested by their expression of molecules such as PD-1 and Lag3. In the former, we can develop ways to manipulate them for therapeutic advantage (for example in inflammatory diseases) or transiently block their function, as might be required for effective vaccination. However, if they represent a terminally differentiated state, then different approaches may have to be devised to either promote or inhibit their development.

OTHER MECHANISMS OF Th1 CELL REGULATION DURING PROTOZOAN INFECTIONS

Although IL-10 is a potent regulator of Th1 cell responses, there is likely to be multiple mechanisms to control such potentially damaging inflammatory responses. Type I IFNs have recently emerged as import immune regulators during parasitic infections. They are produced by most cell types and play critical roles in anti-viral immunity (98, 99), but several studies have identified this family of cytokines as important determinants of disease outcome in protozoan infection. However, these effects depend on the virulence of the parasite and the stage of infection. For example, in

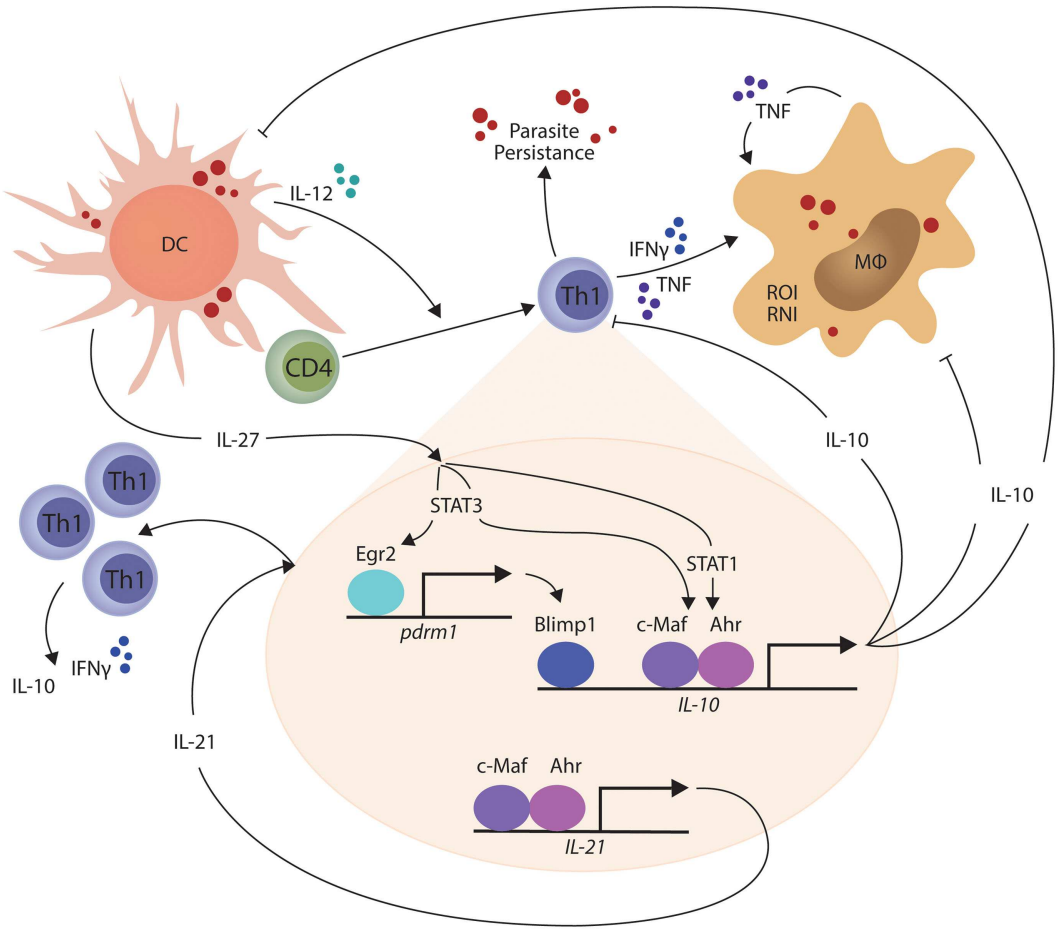

FIGURE 1 | IL-27-mediated generation of IL-10-producing Th1 cells is shown. In the presence of persistent parasite antigen exposure, IL-27 from macrophages and dendritic cells (DCs) stimulates STAT1 and STAT3-dependent transcription of c-Maf and aryl hydrocarbon receptor (AhR) in Th1 cells, which then physically associate and bind the IL-10 and IL-21 gene promoters to drive gene transcription. IL-27 and T cell receptor signaling also combine to promote the expression of the transcription factor Erg2, which is critical for the induction of Blimp1 and generation of IL-10-producing Th1 cells in a STAT3-dependent manner. IL-21 acts as an autocrine growth factor for IL-10-producing Th1 cells, while the IL-10 produced by these cells can suppress the inflammatory functions of Th1 cells and phagocytes, as well as the antigen presenting capacity of DCs, macrophages (MØ), and monocytes (MO). The small red circles represent protozoan parasites and associated antigens.

mice lacking type I IFN receptor, early control of *T. brucei* was impaired, but it appeared that IFNγ production later in infection was suppressed by type I IFN signaling pathways (100). In contrast, early control of a high dose *T. cruzi* infection was enhanced in type I IFN receptor-deficient mice and this was associated with increased IFNγ production, but not when a lower parasite dose was used (101). In liver stage *P. berghei* ANKA infection, parasite RNA triggers a type I IFN transcriptional program in hepatocytes that enhances innate immune responses in hepatic myeloid cells to control liver parasite load (102). In contrast, in mice infected with *P. berghei* ANKA blood-stage parasites, type I IFNs promoted susceptibility to severe disease (103, 104) by suppressing Th1 cell development (103) indirectly through inhibition of $CD8^-$ DC function (105). Interestingly, this latter effect of type I IFN on DC function was also associated with reduced IL-10 mRNA accumulation in $CD8^-$ DCs that lacked type I IFN receptor, potentially linking infection-induced IL-10 production with Th1 regulation once again (**Figure 2**).

TGFβ has also emerged as an important regulatory cytokine controlling Th1 responses during protozoan infections [reviewed in Ref. (106)]. In mice, susceptibility to lethal *P. berghei* ANKA infection correlated with reduced TGFβ levels (107), while high levels of TGFβ in malaria patients was associated with increased parasite growth. In mice infected with *T. gondii*, TGFβ produced by gut intraepithelial $CD8^+$ T cells was critical for controlling inflammation and gut pathology (108), thus supporting a key role for this cytokine in regulating inflammation during protozoan infections. In both malaria and toxoplasma, it is likely that TGFβ acts by suppressing T cell activation and promoting Treg cell functions (109). However, this is yet to be formally demonstrated and there still remains much to learn about the functions of TGFβ during infection.

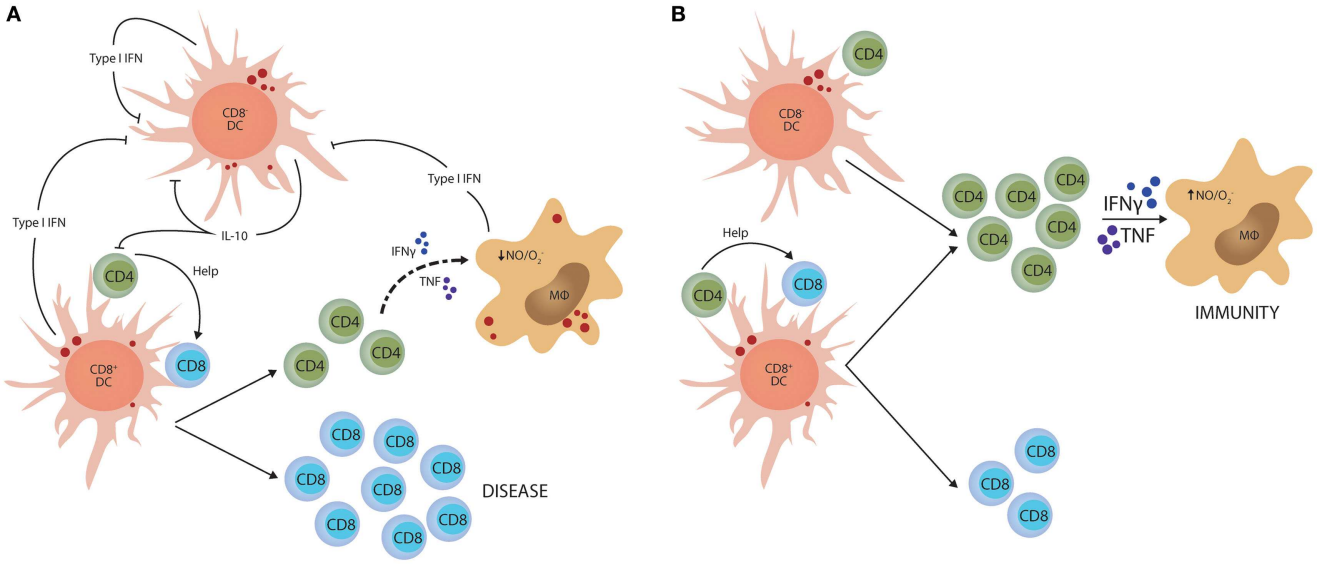

FIGURE 2 | Type I IFN-mediated suppression of Th1 cell activation. **(A)** Parasite molecules stimulate type I IFN production by different dendritic cell (DC) subsets and macrophages (MØ). This family of cytokines feedback on these innate immune cells and suppress their capacity to activate $CD4^+$ T cells. In experimental malaria caused by *Plasmodium berghei* ANKA, this effect is primarily directed toward the $CD8^-$ DC subset and stimulates IL-10 production. **(B)** In this model, blockade of type I IFN signaling dramatically enhances the generation of anti-parasitic $CD4^+$ T cell responses that can protect mice from $CD8^+$ T cell-mediated severe disease. The small red circles represent protozoan parasites and associated antigens.

Both lipoxin A4 and glucocorticoids have also been identified as important regulators of Th1 cell responses in mice infected with *T. gondii* (110–112). Lipoxin A4 is an eicosanoid mediator capable of suppressing DC IL-12 production in response to parasite antigen *in vitro* (110) or during *T. gondii* infection in mice (111). In this latter study, infection of mice lacking lipoxin A4 resulted in a fatal, parasite-induced inflammation (characterized by a potent Th1 cell response), but reduced parasite loads. Importantly, results from this and previous studies (113), suggest that IL-10 was critical for regulating inflammation during the acute stage of infection, while lipoxin A4 was important for immune regulation during chronic infection (111). A novel, IL-10-independent pathway of immune regulation was also recently identified in this infection model, whereby glucocorticoids produced by the hypothalamic–pituitary–adrenal axis during *T. gondii* infection act directly on $CD4^+$ T cells to prevent Th1 cell hyperresponsiveness and resulting pathology (112). Given the critical roles for both IL-10-dependent and IL-10-independent pathways in preventing inflammatory diseases associated with protozoan infections, temporal and/or transient blockade of one or the other pathways may be a viable way to enable sufficient pro-inflammatory immunity to control parasite growth, but also leave enough regulatory machinery in place to prevent disease.

CONCLUDING REMARKS

There are currently no vaccines to protect against or treat diseases caused by protozoan parasites. It has proven extremely difficult to generate robust and long-lasting $CD4^+$ T cell responses against the responsible pathogens (3). An important impediment for generating sufficient immunity against these pathogens could, in some cases, be the accompanying regulatory immune response that aims to limit inflammation. Treg cell depletion can dramatically improve candidate malaria vaccine efficacy (114, 115), although as mentioned above, these studies must be interpreted with caution because of the use of anti-CD25 mAb for Treg cell modulation. Nevertheless, the blockade of IL-10 produced by antigen-specific Th1 cells improved anti-parasitic immunity generated by a candidate vaccine directed against *L. major* (116), while studies on *T. gondii* indicated that induction of IL-10-producing Th1 cells following vaccination caused a lethal infection upon secondary exposure to the parasite (117). Although the depletion of Treg cells and/or IL-10-producing Th1 cells is not a viable option for improving vaccine efficacy given the critical roles of these cells in preventing immune-mediated disease, a much better understanding about how regulatory immune responses can be locally and temporarily modulated to enhance vaccine-induced immune responses may be of significant benefit.

The regulation of Th1 cell responses during protozoan infections is clearly important to ensure both sufficient generation of inflammatory mediators to control parasite growth, as well as to prevent excessive production of these molecules in sensitive tissue sites. IL-10 has emerged as an important regulator of these responses, both produced in a highly regulated manner by Th1 cells themselves, as well as parasite-activated innate immune cells. However, IL-10 is not alone in this activity, and alternative mechanisms of Th1 cell regulation have been identified. Our challenge remains to fully define these mechanisms of Th1 cell regulation and to use this knowledge to improve therapeutic options and vaccine efficacy. Research in protozoan infections of both mice and humans is ideally placed to identify broad mechanisms of immune

regulation that are relevant not only to parasitic infections but also for autoimmune and physiological diseases, as well as cancer.

AUTHOR CONTRIBUTIONS

Christian R. Engwerda, Susanna S. Ng, and Patrick T. Bunn all contributed to the planning and research in this paper. Susanna S. Ng conceived and produced both figures, while Christian R. Engwerda and Patrick T. Bunn wrote the paper.

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Plasticity of human CD4 T cell subsets

Jens Geginat*, Moira Paroni, Stefano Maglie, Johanna Sophie Alfen, Ilko Kastirr, Paola Gruarin, Marco De Simone, Massimiliano Pagani and Sergio Abrignani*

Fondazione Istituto Nazionale di Genetica Molecolare "Romeo ed Enrica Invernizzi" INGM, Milan, Italy

Edited by:
Dragana Jankovic, National Institutes of Health (NIH), USA

Reviewed by:
António Gil Castro, University of Minho, Portugal
Toshinori Nakayama, Graduate School of Medicine, Japan

****Correspondence:***
Jens Geginat and Sergio Abrignani, Fondazione Istituto Nazionale di Genetica Molecolare "Romeo ed Enrica Invernizzi" INGM, Via Sforza 35, Milano 20122, Italy
e-mail: geginat@ingm.org; abrignani@ingm.org

Human beings are exposed to a variety of different pathogens, which induce tailored immune responses and consequently generate highly diverse populations of pathogen-specific T cells. $CD4^+$ T cells have a central role in adaptive immunity, since they provide essential help for both cytotoxic T cell- and antibody-mediated responses. In addition, $CD4^+$ regulatory T cells are required to maintain self-tolerance and to inhibit immune responses that could damage the host. Initially, two subsets of $CD4^+$ helper T cells were identified that secrete characteristic effector cytokines and mediate responses against different types of pathogens, i.e., IFN-γ secreting Th1 cells that fight intracellular pathogens, and IL-4 producing Th2 cells that target extracellular parasites. It is now well established that this dichotomy is insufficient to describe the complexity of $CD4^+$ T cell differentiation, and in particular the human CD4 compartment contains a myriad of T cell subsets with characteristic capacities to produce cytokines and to home to involved tissues. Moreover, it has become increasingly clear that these T cell subsets are not all terminally differentiated cells, but that the majority is plastic and that in particular central memory T cells can acquire different properties and functions in secondary immune responses. In addition, there is compelling evidence that helper T cells can acquire regulatory functions upon chronic stimulation in inflamed tissues. The plasticity of antigen-experienced human T cell subsets is highly relevant for translational medicine, since it opens new perspectives for immune-modulatory therapies for chronic infections, autoimmune diseases, and cancer.

Keywords: CD4 T cells, cytokines, differentiation, tissue homing, plasticity

INTRODUCTION

Human $CD4^+$ T cells are critical regulators of the immune system, as drastically demonstrated by HIV-infected individuals that develop susceptibility to opportunistic infections and cancer when virus-dependent depletion reduces $CD4^+$ T cell counts below critical thresholds (1). $CD4^+$ T cells are very heterogeneous in human adults, because they have been generated in response to a high number of different pathogens and belong to a progressively increasing number of different subsets with specialized functions (2). Helper T cell subsets are defined by the production of cytokines and/or the expression of characteristic lineage-defining transcription factors (**Table 1**). Five principal subsets or lineages of $CD4^+$ T cells have been identified so far: T helper (Th)1, Th2, and Th17 cells that target specific classes of pathogens (3–5), regulatory T cells that are required to maintain self-tolerance (6) and follicular helper T cells (T_{FH}) that provide help to B cells for antibody production (7). Heterogeneity is generated upon T cell priming, since naïve T cells have stem-cell-like properties and can differentiate into virtually all different types of effector, memory, or regulatory cells (**Table 1**). Antigen-experienced T cells are less flexible, but many subsets retain some plasticity and can acquire additional cytokine producing capacities upon antigenic re-stimulation, while others appear to be terminally differentiated (8). In some cases, T cell functions can even completely change from helper to regulatory functions (9) or vice versa (10). A caveat of these findings in particular in humans is the enormous heterogeneity of T cells (2), making it difficult to exclude a selective outgrowth of rare pre-existing precursor cells. Several excellent reviews on the plasticity of mouse T cells have been published in recent years (11–13), while human T cell plasticity is less understood, but highly relevant for new therapeutic strategies in immune-mediated diseases (14).

TERMINALLY DIFFERENTIATED TH1 AND TH2 EFFECTOR CELLS: THE TIP OF THE ICEBERG

Seminal studies have established that $CD4^+$ T cells can differentiate into two types of effector cells with different cytokine producing capacities and functions in humans and mice (3, 4). Uncommitted naïve T cells that are activated by specialized dendritic cells that produce IL-12 (15, 16) acquire IFN-γ producing capacities. These so-called T helper 1 cells (Th1) are induced upon infections with intracellular pathogens like bacteria or viruses and can activate macrophages to destroy intracellular bacteria. In contrast, naïve T cells primed in the presence of IL-4 undergo a different fate and start to produce IL-4, IL-5, IL-10, and IL-13, but not IFN-γ. These Th2 cells are required to fight extracellular parasites like helminths, but since they induce IgE from B cells they are also involved in allergies (17). Importantly, it was shown that Th1 versus Th2 differentiation was a crucial decision to resist infections, since BL/6 mice that mount a Th1 response to leishmania were protected, while BALB/c mice that instead induce a Th2 response were highly susceptible (18). The characteristic cytokines produced by Th1 and Th2 cells, IFN-γ, and IL-4, were further shown to inhibit the differentiation to the opposite differentiation lineage and thus

Table 1 | Phenotype, characteristics and functions of relevant human T cell subsets.

T cell subset	Phenotype	Characteristic cytokines	Characteristic transcription factors	Function
Naïve	$CD45RA^+CCR7^+$	IL-2		Precursor cells, protection against new pathogens
T_{CM} (central memory)	$CD45RA^-CCR7^+$	IL-2, IL-21		Secondary expansions, help
T_{EM} (effector memory)	$CCR7^-$	IFN-γ, IL-4, IL-5, IL-17		Protection in tissues, help
T_{RM} (tissue-resident memory)	$CD103^+CD69^+$	IFN-γ		Immediate protection in tissues
T_{FH} (follicular helper)	$CXCR5^+ICOS^+$	IL-21	BCL6	B cell help
Th1	$CXCR3^+$	IFN-γ	T-bet	Protection against intracellular pathogens
Th2	$CRTH2^+$	IL-4, IL-5, IL-13	GATA-3	Protection against extracellular parasites
Th9	?	IL-9	PU.1	Protection against extracellular parasites
Th17	$CCR6^+CD161^+$	IL-17, IL-22, IL-26	RORC2	Protection against extracellular bacteria and fungi
Treg	$CD25^+CD127^-$	TGF-β	FOXP3	Maintenance of self-tolerance
Tr1 (type 1 regulatory)	$CD25^-CD127^-$ or $CD49b^+LAG3^+$	IL-10	?	Inhibition of immunopathology

reinforced the original fate decision. The capacity to produce either IFN-γ or IL-4 is stably imprinted by epigenetic modifications like DNA methylation and histone acetylations, ensuring that the cytokine profile of T helper cells is preserved upon cellular division independently of the inducing polarizing cues (19–21). Moreover, the generation of Th1 and Th2 cells was shown to depend on the "master" transcription factors T-bet and GATA-3, which induced not only the characteristic cytokines of Th1 and Th2 cells, but also inhibited the differentiation to the alternative lineage. Based on this evidence, it was initially assumed that the differentiation to Th1 and Th2 cells are mutually exclusive and irreversible fate decisions.

TH1 AND TH2 CELLS CAN ACQUIRE NEW PROPERTIES AND FUNCTIONS IN SECONDARY OR CHRONIC IMMUNE RESPONSES

Early studies with human T cell clones showed that IFN-γ and IL-4 production were not necessarily two exclusive features, since some T cells co-produced IFN-γ and IL-4 (22). Notably, human Th1 memory cells are responsive to IL-4 stimulation, and acquire IL-4 producing capacities upon TCR stimulation in the presence of IL-4 without losing IFN-γ production *in vitro* (23).

In addition, some T cells in human blood co-express the Th1 and Th2 markers CXCR3 and CCR4 (24) or CRTh2 as well as the lineage-defining transcription factors GATA-3 and T-bet (25). Consistently, it was shown in mice that histones of these transcription factor genes had both repressive and permissive marks in opposing T cell lineages (13, 26). In mice, *in vivo* primed Th2 cells can acquire IFN-γ producing capacities in addition to IL-4 in response to IFN and IL-12 (27), while human blood Th2 cells seem to be less plastic (23). Moreover, the pathogens and the physiological conditions that induce Th1/2 cells in humans and their role in immune responses remain to be fully defined (25).

Another early finding that did not fit well into the fixed Th1/Th2 paradigma was the fact that IL-12 could induce IL-10 in Th1 cell clones (28). IL-10 has potent anti-inflammatory functions and inhibits maturation and T cell stimulatory capacities of APC (29), thus the concomitant expression of both IFN-γ and IL-10 by T cells was unexpected (30). Later it was shown that IL-10 produced by T-bet$^+$ Th1 cells was required to inhibit lethal immunopathology upon infections with intracellular parasites (31, 32), indicating that IL-10-producing Th1 cells prevent overshooting immune responses and the resulting tissue damage in a negative feedback loop (9). Interestingly, although these IL-10 producing Th1 cells inhibited IL-12 production by APC, they were also able to restrict parasite growth via IFN-γ (31). However, IFN-γ has also been shown to have some negative effects on T cell responses (33, 34), providing a possible alternative explanation for IFN-γ production by regulatory T cells. Importantly, IFN-γ/IL-10 co-producing T cells with regulatory functions are present at low frequencies in peripheral blood of healthy donors and respond selectively to persistent pathogens (35), suggesting that similar to their mouse counterparts they inhibit overshooting immune responses in chronic infections. Thus, Th1 cells can switch from pro-inflammatory effector cells to IL-10 producing type 1 regulatory (Tr1)-like T cells (36, 37), and this switch is necessary to maintain the integrity of infected tissues in some infections. Complement receptor stimulation (38), production of IL-27 (39) or IL-12 (28) by myeloid cells (40), or generation of AHR ligands (41) are possible inductive cues, but also chronic or repetitive antigenic stimulation seems to be required to induce IL-10 production in Th1 cells (35, 42, 43). Interestingly, a recent paper suggests that IL-10/IFN-γ co-producing T cells can also be generated from Th17 cells under the influence of IL-12 or IL-27 in mice (44). If IFN-γ/IL-10 co-producing regulatory T cells are stably maintained or are short-lived, if they progressively lose IFN-γ production upon chronic stimulation or revert to Th1 cells upon pathogen clearance is currently unclear (**Figure 1**).

More recently, additional plasticity of Th2 cells was documented. Thus it was shown that T_{FH} cells were derived from Th2 precursor cells in mouse models of helminth infections (45). This finding is relevant for Th2 stability, because T_{FH} cells are professional B helper T cells that secrete IL-21 in B cell follicles, express the transcriptional repressor BCL-6 and are thus distinct from conventional Th1 and Th2 cells (7, 46, 47). Also in human tonsils a fraction of T_{FH} cells express the Th2 marker CRTH2 and

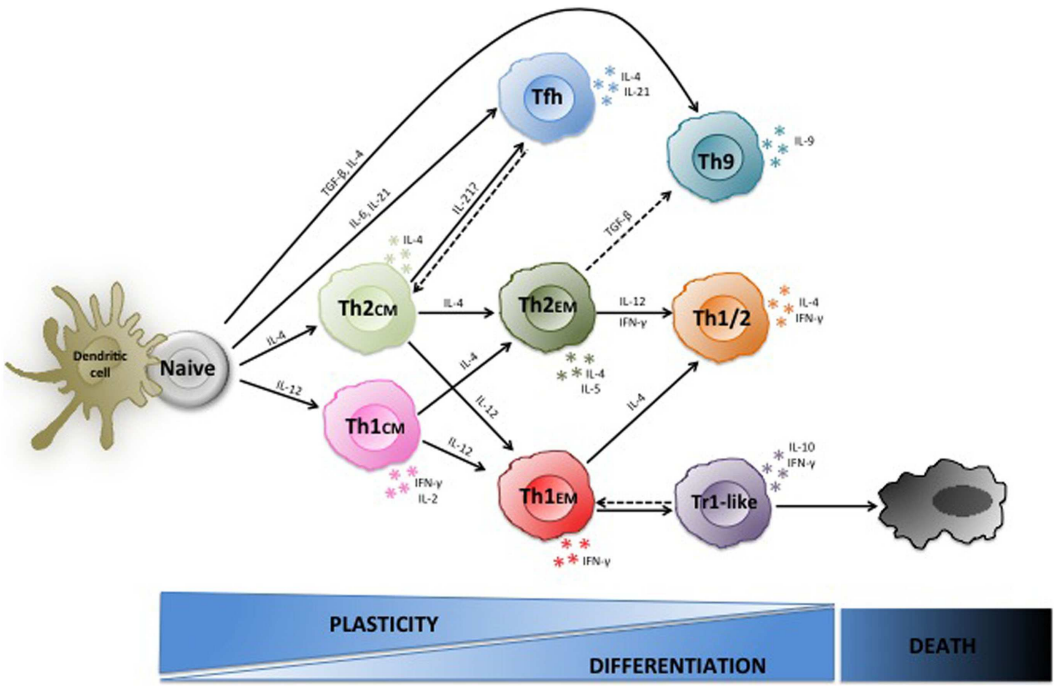

FIGURE 1 | Plasticity of human Th1 and Th2 cells. Naive CD4$^+$ T cells are stem-cell-like cells that under the influence of different cytokines can differentiate to various types of effector cells including Th1, Th2, Th9, and T_{FH} cells. Th1 and Th2 central memory cells are arrested at an early stage of differentiation, are highly plastic and some can still switch lineage. Conversely, effector memory cells are more differentiated, less plastic, and rather become polyfunctional. Moreover, Th1 effector cells can acquire IL-10 producing capacities and regulatory functions in chronically inflammed tissues.

produce IL-4 (48). The relationship of Th1 cells with T_{FH} cells is less clear in particular in humans (49, 50). Some murine T_{FH} cells produce IFN-γ (51), which induces IgG2a production by B cells (52), but T_{FH} cells from human tonsils lack IFN-γ production.

Mouse Th2 cells can also switch from IL-4 to IL-9 production upon stimulation with TGF-β (53). These Th9 cells express the PU.1 transcription factor (54) and can also be directly induced from naïve and memory T cells upon stimulation with TGF-β and IL-4 in humans and mice (55, 56). Th9 cells can have a pro-inflammatory role in allergic asthma (57) and respond to helminth antigens and allergens in humans (58, 59). However, IL-9 induction by TGF-β is not restricted to Th2 cells (60).

Collectively, these findings indicate that both Th1 and Th2 cells can acquire different cytokine producing capacities and functional properties upon antigenic re-stimulation under the influence of cytokines, and are thus much more flexible than originally thought (**Figure 1**).

STABILITY OF FOXP3$^+$ TREGS IS DEBATED

CD25$^+$ regulatory T cells are required to maintain self-tolerance. They were first identified in mice (61) and later in humans (62), and the Foxp3 transcription factor was shown to be required for their generation and function (63, 64). Consistently, IPEX patients, who suffer from a devastating autoimmune disease, were found to have mutations in the Foxp3 gene (65). Although so-called natural or thymic Foxp3$^+$ Tregs acquire regulatory lineage commitment already upon maturation in the thymus (66), adaptive, or peripheral Foxp3$^+$ Tregs can be induced from mature CD4$^+$ helper T cells in the periphery under the influence of TGF-β (67, 68). The transcription factor Helios was proposed to distinguish between these two subsets of natural and induced Foxp3$^+$ Treg, but this concept was not confirmed by others (69–71). In humans, CD45RA$^+$CD25$^+$Foxp3$^+$ cells represent a population of *bona fide* "naïve" and thus thymus-derived Tregs, while CD45RA$^-$ Tregs are a mixed population that contain antigen-experienced Tregs of both thymic and peripheral origin (72). The stability of Foxp3$^+$ Tregs is debated (73). Lineage tracing of Foxp3$^+$ T cells in mice has lead to conflicting interpretations, since in several studies only very small fractions of Foxp3$^+$ Tregs were found to lose Foxp3 and regulatory functions *in vivo* (74). In humans, CD45RA$^+$ but not CD45RA$^-$ Tregs could be stably expanded *in vitro* (72, 75), suggesting different stabilities of thymic and peripheric Tregs. However, since human Tregs have to be purified according to surface marker expression, it is difficult to exclude a selective outgrowth of Foxp3$^-$ cells or of activated effector T cells that have transiently up-regulated Foxp3 upon stimulation (73).

The functional specialization of Foxp3$^+$ Treg is shaped by the tissue microenvironment (76), and the induction of transcription factors characteristic for helper T cell lineages in mice allows Tregs to suppress the corresponding T helper cell responses (74). Thus, STAT3 in Tregs is required to suppress Th17 cells (77), IRF4 to control Th2 responses (78) while Tregs that regulate T_{FH} cells and antibody responses express BCL-6 (79, 80). Foxp3$^+$ Tregs also acquire T-bet and IFN-γ producing capacities upon

stimulation with IL-12, and these Th1regs might be specialized to suppress Th1 responses (14, 74). Tregs also inhibit anti-tumor CTL responses (81), and interestingly they can acquire cytotoxic properties in tumor-draining lymph nodes in mice (82) and *in vitro* in humans (83), and tumor-infiltrating Tregs are consequently cytotoxic (84). Similar to helper T cells, Tregs that secrete different types of effector cytokines can be identified according to chemokine receptor expression (2), and these Treg subsets might specifically suppress different types of immune responses (85). Human Foxp3$^+$ T cells that produce IL-17 or IFN-γ can be isolated (86, 87), but while IL-17 producing Treg cells were normally suppressive (88), IFN-γ producing Tregs had reduced suppressive functions (87). The conditions that induce human Foxp3$^+$ Tregs to secrete different effector cytokines and the role of these cells in infections, cancer, and autoimmune diseases remain to be fully established.

HETEROGENEITY AND UNSTABILITY OF TH17 CELLS AND ITS RELEVANCE FOR AUTOIMMUNE DISEASES

The discovery of IL-17 producing helper T cells (Th17) in mice (89, 90) and humans (91) and their relative instability (11, 92) has led to a profound re-evaluation of the concept of two terminally differentiated helper T cell subsets. The fact that human CD4$^+$ T cells produce IL-17 was known for a long time (93). However, it took a decade to realize that these cells represented an independent differentiation lineage (89, 90), which have unique differentiation requirements and express the lineage-defining transcription factor ROR-γt in mice and RORC2 in humans (94, 95). Th17 cells are important to fight extracellular bacteria and fungi, since patients that lack Th17 cells have uncontrolled infections with *Candida albicans* (*C. albicans*) and *Staphylococcus aureus* (96). The discovery of Th17 cells has been complicated by the fact that T cell differentiation to Th1 and Th17 cells relies on shared components of cytokines and their receptors. Thus, it was known that IL-12p40 and IL-12Rβ1 hetero-dimerize with respectively IL-12p35 and IL-12Rβ2 to induce Th1 cells, but later it was realized that they can also associate with respectively IL-23p19 and the IL-23R to promote Th17 responses (97). The IL-23/IL-23R pathway is involved in many different autoimmune diseases (98–100) and IL-23-induced Th17 cells are thought play a prominent pathogenic role (101–104). Conversely, the contribution of Th1 cells, which were initially thought to drive autoimmune diseases, is now debated. The requirements for Th17 differentiation are more complex than for Th1 and Th2 cells, because IL-17 production in CD4$^+$ T cells can be induced by different cytokine combinations. Initially, TGF-β plus IL-6 was identified in mice (105), while IL-1β, IL-6, and/or IL-23 were proposed in humans (106, 107). The *de novo* Th17 differentiation is very inefficient in humans, and therefore it was suggested that only a cocktail with all four cytokines induces significant Th17 differentiation (108). Although the role of TGF-β in human Th17 differentiation has been a subject of debate (109), it was shown in mice that TGF-β induces ROR-γt, while pro-inflammatory cytokines are required to inhibit TGF-β-induced Foxp3 expression and thus Treg generation (110). The presence of CD4$^+$ T cells co-expressing Foxp3, RORC2, and/or IL-17 in humans is consistent with a role for TGF-β in human Th17 and Treg development (86, 88). An alternative explanation for the positive role of TGF-β in Th17 differentiation is that TGF-β indirectly favors Th17 cell differentiation by inhibiting Th1 cell development (111). Indeed, in the absence of TGF-β1 (106, 107, 112), or in the presence of TGF-β3 in mice (113), pathogenic Th17 cells that co-produce IL-17 and IFN-γ are generated. These Th1/17 cells co-express RORC2 and T-bet, are enriched in autoimmune patients and are specific for both Th1 and Th17-inducing pathogens (114, 115).

In vitro stability experiments and fate reporter mice suggested that Th17 cells are partially unstable and can switch completely from IL-17 producing Th17 to IFN-γ producing Th1 cells in chronic immune responses (92, 116). IL-12 can induce this Th17-to-Th1 switch (117), and CD161 was proposed as a marker that distinguishes these ex-Th17 cells from conventional Th1 cells in humans (118). However, *ex vivo* isolated human Th17 cells exhibited stable epigenetic marks at cytokine and transcription factor loci (119), suggesting that *in vivo* generated human Th17 cells are not necessarily unstable. Finally, also a very rare population of human T cells that co-produces IL-17 and IL-4 was identified (120). These Th2/17 cells were proposed to be highly pro-inflammatory in allergic asthma, but their role in immune responses against pathogens remains to be understood.

Th17 cells are highly heterogeneous and produce several effector cytokines besides IL-17. IL-22, a cytokine that promotes epithelial proliferation and barrier function (121), is produced by some Th17 cells (122, 123), and IL-22 and IL-17 co-operate to control gram-negative bacteria in the lung (124). However, a subset of human skin-homing IL-22 producing cells was identified that were distinct from Th17 cells (125, 126). Indeed, in contrast to IL-17, IL-22 is inhibited by TGF-β (127) and thus how Th17 cells acquire IL-22 producing capacities and if they can even switch from IL-17 to IL-22 production is unclear. Some Th17 and Th22 cells also produce IL-26, a pro-inflammatory cytokine that is not expressed in mice (128) and that also acts selectively on non-hematopoietic cells. A particular relevant cytokine in the pathogenesis of experimental autoimmunity is GM-CSF, which is induced by IL-1β, IL-23, and ROR-γt in mice (102, 129). Conversely, GM-CSF is inhibited by IL-1β and IL-23 in humans, and is produced by both Th1 and Th17 cells (130, 131).

Th17 cells also produce high levels of IL-21. IL-6 induces IL-21 in naive T cells upon priming (132), and IL-21 can induce its own expression (133) and promotes Th17 differentiation in an autocrine manner (131, 134–136). Importantly however, IL-21 inhibits GM-CSF and IFN-γ production and promotes instead IL-10 secretion in developing Th17 cells. Consequently, IL-21 promotes the generation of conventional (137) or regulatory Th17 cells (138), but inhibits the generation of pathogenic Th1/17 cells (131). Finally, a subset of skin-homing T cells produces IL-9 and responds to *C. albicans* (139). Some of these cells co-produce IL-9 and IL-17 (60), while others appear to represent Th9 cells. IL-9 production seems however to be transient, suggesting that these skin-homing Th9 cells are largely unstable (139).

In summary, the current knowledge indicates that human Th17 cells are highly heterogeneous and partially unstable (**Figure 2**), and much remains to be learned on the role of different Th17 subsets in immune-mediated diseases.

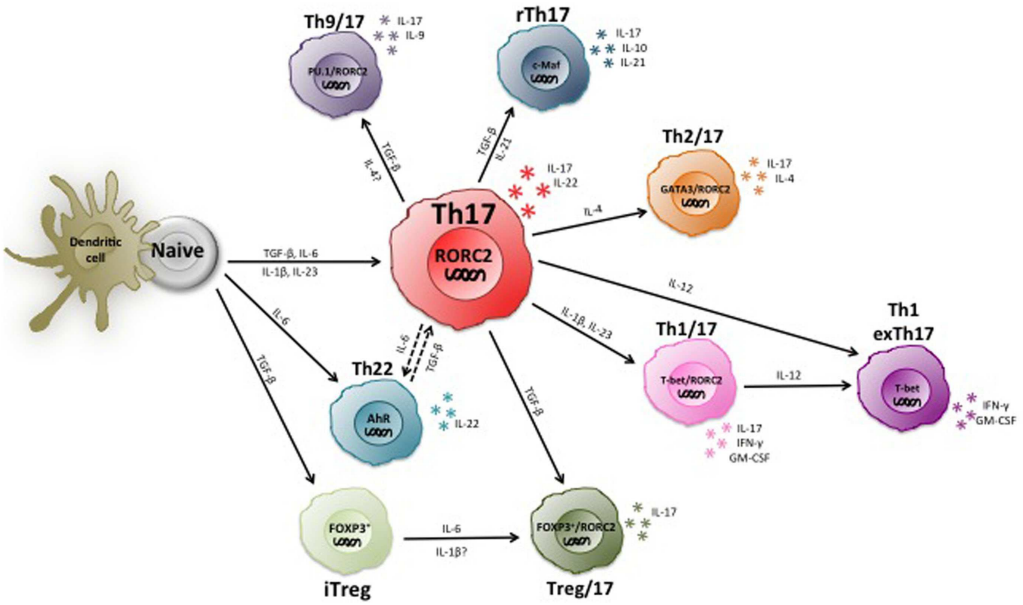

FIGURE 2 | Heterogeneity and plasticity of human Th17 cells. Th17 cells are highly heterogeneous and produce various types of other cytokines in addition to IL-17, including the Th1 and Th2 marker cytokines IFN-γ and IL-4. Some IL-17 producing T cells express Foxp3 and/or IL-10 and are suppressive. Moreover, Th17 cells are partially unstable and can become Th1 cells upon chronic inflammation.

REGULATION OF HUMAN T CELL PLASTICITY IN TISSUES: THE NEW FRONTIER

The complex regulation of T helper subsets by cytokines raises the questions where T cells are re-educated and also why this might be important to successfully resist pathogens, since this was a major evolutionary pressure that shaped the human immune system. It was soon realized that stable T cell differentiation often requires repetitive *in vitro* TCR stimulation in the appropriate cytokine condition, suggesting that immature T cells might be more plastic than more differentiated ones (12, 140). *In vivo* primed T cells that are at an intermediate stage of differentiation are central memory T cells (T_{CM}), which similar to naïve T cells have maintained the capacity to home to lymph nodes, produce only low levels of effector cytokines, but produce high levels of IL-2 and IL-21 (131), and expand rapidly to generate secondary waves of effector cells (8). Conversely, effector memory T cells (T_{EM}) are more differentiated cells since they produce high levels of effector cytokines and home preferentially to inflamed non-lymphoid tissues (8). Consistent with the view that plasticity is progressively reduced upon T cell differentiation, pre-committed $Th1_{CM}$ cells are more plastic than fully differentiated $Th1_{EM}$ cells, since $Th1_{CM}$ cells generate a substantial population of *bona fide* Th2 cells upon re-stimulation with IL-4, while $Th1_{EM}$ cells do not revert to Th2 cells, but some acquire IL-4 in addition to IFN-γ producing capacities (24). This plasticity requires TCR stimulation, since antigen-independent proliferation with homeostatic cytokines resulted exclusively in the generation of Th1 effector cells (24). Based on these findings it can be speculated that pre-committed T_{CM} cells that cross-react with a different pathogen can be still partially re-educated to a different lineage in lymph nodes, while T_{EM} cells do not easily switch cytokine production, but rather become polyfunctional (**Figure 1**). Another example of functional plasticity in lymphoid organs is the generation of follicular $Foxp3^+BCL\text{-}6^+$ Tregs, which are specialized Tregs that control B cell responses (79, 80). Also Tregs in non-lymphoid tissues acquire tissue-specific properties that are important for their functions (76). T_{EM} helper cells that are activated by antigen in non-lymphoid tissues can up-regulate CCR7 (141) and home to inflamed lymph nodes (142) where they can influence the secondary immune response and are exposed to a different cytokine milieu. Conversely, tissue-resident memory (T_{RM}) cells have lost sphingosine-1 phosphate receptors and thus also the capacity to re-circulate through the blood to secondary lymphoid organs (143). T_{RM} belong predominantly to the CD8 compartment, but influenza virus-specific $CD4^+$ T_{RM} can be identified in the lung of humans and mice (144). If tissue-resident $CD4^+$ T cells are terminally differentiated effector cells or still possess the plasticity to acquire additional cytokine producing capacities remains to be established (145).

A central organ for the generation of different subsets of Th17 cells is the intestine (146). Thus, upon self-limiting colitis induced by anti-CD3 injections in mice predominantly IL-10 producing Th17 cells with regulatory functions are induced (138). Conversely, under conditions that induce IL-23 in the intestine pathogenic IFN-γ and GM-CSF producing Th17 cells are generated that induce colitis (147, 148). IFN-γ and IL-17 co-producing Th1/17 cells have also been observed in patients with IBD (92), but very little is known about the regulation of Th17 responses in the human intestine. Th1/17 cells that produce IL-17, IFN-γ, and GM-CSF also drive central nervous system (CNS) inflammation in EAE, a standard mouse model of multiple sclerosis (MS) (149). The CNS is separated from pro-inflammatory T cells by the blood–brain barrier (150), but spontaneous JC Virus

re-activations and progressive multifocal leukoencephalopathy in MS patients treated with anti-VLA-4 antibodies, which block lymphocyte extravasation to the CNS, suggest nevertheless a constant immune surveillance by T cells (151). How the microenvironment of the CNS influences the properties of $CD4^+$ T cells is the focus of intensive research in mice, but is largely unknown in humans given the difficulties to analyze T cells in the human CNS.

Thus, accumulating evidence underlines the role of the tissue microenvironment in T cell plasticity, and the identification of tissue-specific factors that control T cell functions is likely to have a major impact on translational medicine.

CONCLUSION AND PERSPECTIVE

The original concept of two terminally differentiated subsets of Th1 and Th2 cells has been substituted by the view that many different T cell subsets with specific cytokine profiles are required to protect us from the different pathogenic insults that were are continuously exposed to. These various T cell subsets possess different degrees of plasticity to acquire new characteristics and functions in secondary or chronic immune responses. In particular, while the stability of Tregs is debated, it is widely accepted that Th17 cells are largely unstable, although exceptions might exist. In addition, human Th17 cells are highly heterogeneous, but the functions of all these different types of Th17 effector cells in protective immune responses and their roles in autoimmune diseases remain to be understood. Another important but poorly understood aspect of T cell plasticity is how different tissue microenvironments impact on human T cell differentiation and stability. The definition of the relative plasticities or stabilities of human T cell subsets in different tissues is highly relevant for future therapeutic interventions in so different immune-related pathologies as chronic viral infections, cancer, and autoimmune diseases.

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Long intergenic non-coding RNAs: novel drivers of human lymphocyte differentiation

Ilaria Panzeri*[1], *Grazisa Rossetti*[1], *Sergio Abrignani*[1] *and Massimiliano Pagani*[1,2]

[1] *Integrative Biology Unit, Istituto Nazionale Genetica Molecolare "Romeo ed Enrica Invernizzi", IRCCS Ospedale Maggiore Policlinico, Milano, Italy*
[2] *Department of Medical Biotechnology and Translational Medicine, Università degli Studi di Milano, Milano, Italy*

Edited by:
Dragana Jankovic, National Institutes of Health (NIH), USA

Reviewed by:
Christopher E. Rudd, University of Cambridge, UK
John J. Miles, Queensland Institute of Medical Research, Australia
Susan Carpenter, University of California San Francisco, USA

***Correspondence:**
Massimiliano Pagani, Integrative Biology Unit, Istituto Nazionale Genetica Molecolare "Romeo ed Enrica Invernizzi", IRCCS Ospedale Maggiore Policlinico, via Francesco Sforza 35, Milano 20122, Italy
e-mail: pagani@ingm.org

Upon recognition of a foreign antigen, $CD4^+$ naïve T lymphocytes proliferate and differentiate into subsets with distinct functions. This process is fundamental for the effective immune system function, as $CD4^+$ T cells orchestrate both the innate and adaptive immune response. Traditionally, this differentiation event has been regarded as the acquisition of an irreversible cell fate so that memory and effector $CD4^+$ T subsets were considered terminally differentiated cells or lineages. Consequently, these lineages are conventionally defined thanks to their prototypical set of cytokines and transcription factors. However, recent findings suggest that $CD4^+$ T lymphocytes possess a remarkable phenotypic plasticity, as they can often re-direct their functional program depending on the milieu they encounter. Therefore, new questions are now compelling such as which are the molecular determinants underlying plasticity and stability and how the balance between these two opposite forces drives the cell fate. As already mentioned, in some cases, the mere expression of cytokines and master regulators could not fully explain lymphocytes plasticity. We should consider other layers of regulation, including epigenetic factors such as the modulation of chromatin state or the transcription of non-coding RNAs, whose high cell-specificity give a hint on their involvement in cell fate determination. In this review, we will focus on the recent advances in understanding $CD4^+$ T lymphocytes subsets specification from an epigenetic point of view. In particular, we will emphasize the emerging importance of non-coding RNAs as key players in these differentiation events. We will also present here new data from our laboratory highlighting the contribution of long non-coding RNAs in driving human $CD4^+$ T lymphocytes differentiation.

Keywords: long non-coding RNAs, epigenetic regulation, lymphocyte differentiation

THE REVOLUTIONS OF REGULATORY NON-CODING RNAs

At the beginning of this century, the results of the human genome project highlighted the complexity of our genome. What emerged was that the fraction of the genome that is informative is higher than we expected. Subsequent analysis revealed that the vast majority of informative sequences does not encode for proteins. Indeed against a total of 62.1% of the human genome covered by processed transcript (74.7% by primary transcripts), exons of protein-coding genes cover only the 2.94% of the genome (1). From an evolutionary point of view, the genome size is in close relationship with coding potential in prokaryotes, which have haploid genomes primarily composed by protein-coding sequences (~88%). Conversely, in eukaryotes, a correlation lacks between protein-coding gene number and organismal complexity. These observations are likely explained by the evolution of a more sophisticated architecture to control gene expression that includes the expansion of non-coding regulatory RNAs (ncRNAs) (2). Thus, we should clearly reassess the centrality of protein-coding RNAs in favor of non-coding ones.

Non-coding RNAs with fundamental functions within cells are known since the discovery of the first transfer RNA (tRNA) (3) and comprise also ribosomal RNAs (rRNAs). Nonetheless, the interest toward non-coding RNAs with regulatory functions arose with the discovery of the first human micro-RNA, let-7 (4). In order to apply a theoretical framework to the transcriptome, regulatory ncRNAs are usually classified based on their dimension: "small" ncRNAs being less than 200 nucleotides in length and "long" or "large" ncRNAs (lncRNAs) ranging from more than 200 to tens of thousands of nucleotides (**Table 1**).

Further complicating the picture, lncRNAs seem to be the preferred substrate for the generation of small RNAs (21). This maze of non-coding transcripts was revealed also in a genome-wide identification of lncRNAs in mouse $CD8^+$ T lymphocytes, where 18 of the identified lncRNAs appeared to overlap with annotated miRNAs and 21 with snoRNAs (37).

Both classes can be further classified according to their position relative to known sequences of the genome, like in the case of promoter-associated RNAs (PASRs) or transcription initiation small RNAs (tiRNAs). In particular, long non-coding RNAs are usually classified relative to neighboring protein-coding genes. They can be defined as "sense" if they are transcribed from the same strand of the protein-coding gene or "antisense" if the opposite is true. They can be "divergent" if their promoter and the one of the coding transcript are in close proximity and located in a head to head fashion. They can be "exonic" or "intronic" if they overlap one or more exons, or an intron of the protein-coding

Table 1 | Major classes of short and long regulatory non-coding RNAs.

ncRNA		Length (nt)	Function
SHORT			
miRNAs	Micro RNAs	21–23	In animals, associate with the miRNA-induced silencing complex (RISC) and silence the expression of target genes mostly post-transcriptionally (5–7)
snoRNAs	Small nucleolar RNAs	60–300	Help the chemical modification of mRNAs, thereby influencing stability, folding, and protein-interaction properties (8, 9)
snRNAs	Small nuclear RNAs	150	Assist splicing of introns from primary genomic transcripts (10, 11)
piRNAs	Piwi-interacting RNAs	25–33	Associate with the highly conserved Piwi family of argonaute proteins and are essential for retrotransposon silencing in germline, epigenetic modifications, DNA rearrangements, mRNA turnover, and translational control also in soma (12–14)
PASRs	Promoter-associated short RNAs	22–200	Enriched at the 5′ end of genes, within 0.5 kb of TSS. Can be transcribed both sense and antisense. Their function and biogenesis is not fully understood (15, 16)
TASRs	Termini-associated short RNAs	22–200	Can be transcribed both sense and antisense near termination sites of protein-coding genes. Their function and biogenesis is not fully understood (15, 16)
siRNAs	Short interfering RNAs	21–23	Processed from a plethora of genomic sources, both foreign (viruses) and endogenous (repetitive sequences). Canonically induce the degradation of perfectly complementary target RNAs (17, 18)
tiRNAs	Transcription initiation RNAs	15–30	Enriched immediately downstream transcriptional start sites (TSSs) of highly expressed genes. Their function and biogenesis is not fully understood (16, 19, 20)
LONG			
NATs	Natural antisense transcripts	>200	Transcribed from the same locus but opposite strand of the overlapping protein-coding sequence. Involved in gene expression regulation, RNA editing, stability, and translation (21, 22)
PALRs	Promoter-associated long RNAs	200–1000	Enriched at promoters, found to regulate gene expression (23, 24)
PROMPTs	Promoter upstream transcripts	200–600	Enriched at TATA-less, CpG-rich promoters with broad TSSs. Affect promoter methylation and regulate transcription (25–27)
T-UCRs	Transcribed ultraconserved regions	>200	Perfectly conserved between human, rat, and mouse. Frequently located at fragile sites and at genomic regions involved in cancer (28)
Intronic RNAs		>200	Transcribed from introns of overlapping protein-coding sequences. Involved in the control of gene expression, alternative splicing, and source for generation of shorter regulatory RNAs (29)
eRNAs	Enhancer-derived RNAs	>200	Function still not completely understood. May functionally contribute to the enhancer function (30–32)
LincRNAs	Long intervening (intergenic) RNAs	>200	Gene expression regulation, regulation of cellular processes (33, 34)
uaRNAs	3′UTR-derived RNAs	<1000	Derive within 3′ untranslated region (3′UTR) sequences. Function still not clearly understood (35)
circRNA	Circular RNA	100 to >4000	Diverse, from templates for viral replication to transcriptional regulators (36)

gene respectively. Instead, they can be "intergenic" (or "intervening"; lincRNAs) if they lie within a sequence between two protein-coding genes (38). In this review, we will focus on this last category, which is probably the most studied given that the location of these lncRNAs avoids complications deriving from the overlap with other genes. The majority of known lncRNAs is generated by the same transcriptional machinery of mRNAs. This means that transcribed lincRNAs genomic sequences are marked by RNA polymerase II occupancy and histone modifications that are shared with active protein-coding genes, such as H3K4me3 at promoters and H3K36me3 within gene bodies (39). They are capped by methylguanosine at their 5′, spliced, and polyadenylated, even if the widespread representation of this last property among known lncRNAs could be partially due to the RNA sequencing strategies used for their identification (15, 40). Indeed, broader analysis identified about 39% of lncRNAs to have at least one of the six most common poly(A) motifs, compared to 51% for coding transcripts (1). These properties imply that there are few distinctive biochemical features that allow the distinction of lncRNAs from protein-coding mRNAs. Among

them, lncRNAs have unusual exon structure, with on average 2–5 exons. Intriguingly, lncRNAs are significantly more likely to overlap repetitive elements and particularly RNA-derived transposable elements (TEs). These last account for about 30% of human lncRNAs nucleotides, often in proximity of their transcriptional start site (TSS), which could suggest that TEs could be important drivers of lncRNAs evolution (see below). Nonetheless, the main difference between lncRNAs and protein-coding genes relies by definition on their coding potential: lncRNAs does not possess open reading frames (ORFs), as evaluated based on: the conservation of ORFs codons (41), ORFs length, the presence of known protein domains, *in vitro* translation (42, 43), and ribosome footprinting (44) assays. However, these conceptual constraints are terribly artificial: short, non-canonical peptides have been found to arise from small ORFs within ncRNA (45–48); lncRNAs genes can also code for proteins and have a double function (49) and ultimately, the coding potential does not necessarily exclude a function as RNA also for known mRNAs (50). Evolution makes boundaries between coding and non-coding genes fainter as ncRNAs can evolve by pseudogenization. This event can follow disruption of the ancestral ORF, but not of the untranslated regulatory regions (UTRs) in protein-coding genes duplicates (50) or can arise without duplication, but from the co-option of ancestral genes to different, non-coding functions (51). This was the case of the long-known Xist RNA, involved in the silencing of the inactive X chromosomes in eutherians. In particular, two exons of the protein-coding gene Lnx3 are homologous to Xist. This gene retained a protein-coding capacity at least in the common ancestor of marsupials and placentals. Conversely, the Xist A-repeat implicated in X-silencing function is not conserved. This sequence likely arose from the insertion of a TE recruited to form a proto-Xist gene (52, 53). Therefore, the difference between dosage compensation in marsupials, eutherians, and monotremes can be ascribed from the presence of a Xist-independent XCI in mammalian ancestor and the peculiar evolution of the proto-Xist gene by pseudogenization in the eutherian ancestor. Intriguingly, other lncRNAs involved in X-inactivation are similarly examples of pseudogenization (54). The boundary between coding and non-coding is even less defined when ncRNAs arise from joining of coding and non-coding exons through alternative splicing (55, 56), from untranslated regions of mRNAs (57, 58) or from the opposite strand of the overlapping protein-coding gene (59). Strikingly, more than a half of protein-coding genes in mammals have a complementary non-coding transcript (60). These findings further challenge our "linear" model of the genome, prompting a re-evaluation of current dogma and genes definitions. Genomic regions indeed are far more complex than previously thought: genes can be used for different purposes and different functional elements can co-locate intermingling coding and non-coding regions.

The interest toward lncRNAs has been rapidly growing and their expressions have been quantitated in many different tissues and cell types by high-throughput sequencing (RNA-seq). These efforts retrieved catalogs with little overlap, so that the number of known lncRNAs is still growing, in contrast with the number of known protein-coding genes that has been remarkably stable over years. Indeed, lncRNAs are far more cell-specific than mRNAs, generally less but also more dynamically expressed at various differentiation stages. For this reason, immune system is an excellent context in which we can deepen our knowledge on lncRNAs. While many excellent reviews cover the recent advances in understanding the role of these molecules within the innate branch (61, 62), little is still known about their importance for the human adaptive immune system. Effector lymphocytes are highly specialized cells that arise from common progenitors through differentiation processes still not completely understood. Besides, lymphocytes can be purified through cell sorting from blood of healthy donors and the existence of *in vitro* differentiation protocols provide the ideal setting for the identification of lncRNAs expressed in the human immune system and for their functional characterization.

Indeed, the growing interest on lncRNAs and the lack of knowledge on their expression patterns in the human immune system prompted us to perform the RNA-seq analysis on 13 human primary lymphocytes subsets purified by FACS sorting from healthy donors ($CD4^+$ naïve, T_H1, T_H2, T_H17, T_{reg}, T_{CM}, T_{EM}, $CD8^+$ naïve, T_{CM}, T_{EM}, B naïve, B memory, B $CD25^+$) and to develop a bioinformatics pipeline for lincRNAs identification.

Through this analysis, we identified long intergenic ncRNAs genes expressed in these subsets and confirmed that lincRNAs cell-specificity is higher than protein-coding genes even when comparing lincRNAs genes with membrane receptor protein-coding genes, which are generally referred as the most accurate markers for lymphocyte subsets definition. Besides, a major outcome of this analysis is the identification through *de novo* transcriptome reconstruction of 563 novel, previously unannotated long intergenic ncRNAs genes, increasing by ~12% the number of lincRNAs known to be expressed in human lymphocytes (63). Intriguingly, a fraction of lincRNAs specific for B cells and a fraction of "pan-T" lincRNAs also exist (63). It would be extremely interesting to study these lincRNAs during lymphocytes development in order to understand their likely peculiar role in thymic or bone-marrow-derived cells development.

These observations imply that the little overlap between available catalogs is a direct consequence of lncRNAs specificity and that we could overcome this limitation only assessing lncRNAs expression in every different, highly purified cell type at different developmental stages, instead of considering tissues as a whole. Moreover, due to their specificity of expression, human lymphocytes lincRNAs that are not yet annotated in public resources would have not been identified without performing *de novo* transcriptome reconstruction. As mentioned before, such tissue-specificity has been linked to the enrichment of TEs in proximity to lincRNAs TSS (64, 65). Moreover, RNA-seq experiments performed in a human $CD4^+$ naïve T cells *in vitro* differentiation time-course suggest that lincRNA-specific expression in human lymphocyte subsets is acquired during their activation-driven differentiation from naïve to memory cells (63).

These findings hint to the involvement of lymphocyte-specific lincRNAs as fine-tuners in cell fate commitment, differentiation, and maintenance of cell identity, as demonstrated by many examples in other cell types (66–68). Also, lincRNAs are functionally involved in cell growth (69, 70), apoptosis (71–74), development (75–77), imprinting (78–80), and dosage compensation (81) in almost every cellular context (**Table 2**).

Table 2 | Examples of lincRNAs with key roles in various cellular contexts.

LncRNA	Cellular context	Function
H19	HSC, placenta	Maternally expressed imprinted gene important for inhibiting placental *growth* (69) and maintaining adult hematopoietic stem cell populations (HSC) via miR-675 generation and repression of Igf1r (70)
GAS5	T lymphocytes, cancers	Plays an essential role in normal *growth arrest* in T lymphocytes (71). Its increased level of expression correlate with cell death and reduced cell proliferation both in prostate (72) and colorectal cancer cell lines. Its lower expression is instead significantly correlated with larger tumor size and poor prognosis in colorectal cancer patients (74)
Linc-MD1	Muscles	Governs the timing of muscle *differentiation* by acting as a competing endogenous RNA (ceRNA) with respect to miR-133 and miR-135 in mouse and human myoblasts (75)
Xist	Somatic cells	Expressed by the future inactive X chromosome, triggers gene silencing *in cis* by coating the chromosome. It induces a cascade of chromatin changes, post-translational histone modification and DNA methylation, and leads to the stable repression of X-linked genes, ensuring proper *dosage compensation* (81)
KCNQ1OT1	Most tissues	Paternally expressed antisense transcript to an interior portion of *Kncq1*, part of an imprinted locus on human chromosome 11p15. It is critical for *imprinting*-mediated silencing in most tissue, via long-range intrachromosomal loop and recruitment of polycomb repressive complex 2 (PRC2) (78–80)

Long non-coding regulatory RNAs exert their function in these fundamental processes interacting with chromatin or DNA modifiers and transcription factors (TFs) modulating gene expression (82); competing with micro-RNAs acting as sponges (83); modulating subcellular trafficking (84), translation (85), splicing (86), and likely through many other mechanisms still to be discovered (**Figure 1**).

Long non-coding regulatory RNAs functional flexibility derive from their intrinsic propensity to fold into thermodynamically stable secondary and higher orders structures that function as interaction modules (87). Each module can fold independently from another, forming bonds at the level of Watson–Crick, Hoogstein, and ribose face (88, 89). These RNAs can rapidly shift between diverse stable structural conformation, allowing allosteric transitions that can act as switches in response to environmental stimuli. They are also processed faster than mRNA, given that they must not be translated, allowing a rapid response to signals. LncRNAs can also be regulated via more than a hundred different nucleotide modifications, like in the case of tRNAs, rRNAs, and snoRNAs (90–92) that modulate their function and probably their structure. RNAs can generate multiple modules within their structure, allowing the interaction with multiple players, the reception of multiple stimuli, and the generation of multiple outputs. The required pairing is likely extremely flexible, such as in the case of micro-RNAs, and allows mismatches, bulges, and wobblings (93). Many of these interaction modules derive from repetitive elements, such as transposons that took advantage of the fewer constraints that lncRNAs sequences have compared to protein-coding genes (1, 94). Indeed, lncRNAs rate of sequence evolution is higher relative to protein-coding genes, even if also these transcripts exhibit evolutionary signatures of functionality. They evolve under modest but detectable selective pressure, accumulating fewer substitutions than neutrally evolving sequences (95, 96). Likely, conservation of relatively small units of lncRNAs sequences (estimated to be less than 5%) could be sufficient to preserve their function, considering their already mentioned modular structure (97). This could be the reason why existing bioinformatic approaches fail to detect low level and scattered selective constraint within these loci (97).

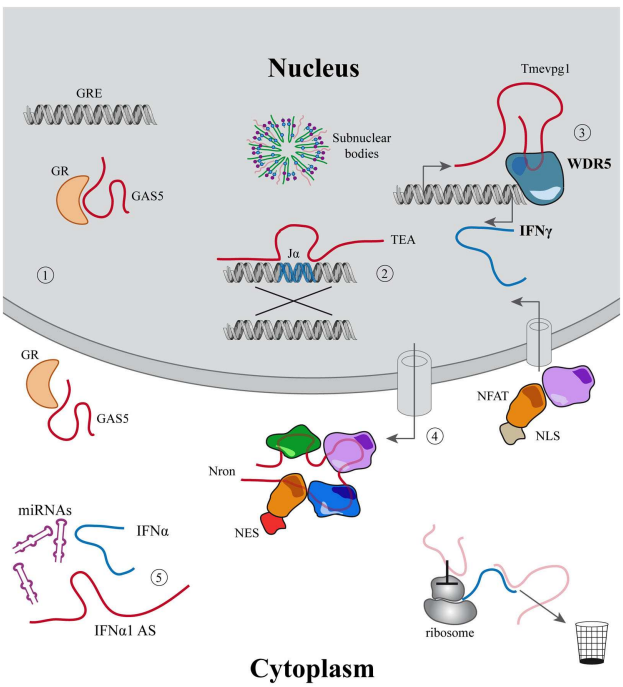

FIGURE 1 | Examples of the main functions associated to lincRNAs. (1–5) LincRNAs described in the immune system. (1) Modulation of cell growth and apoptosis mediated by GAS5 that acts inhibiting glucocorticoid receptors binding to their DNA responsive elements; (2) Jα recombination guided by the PARL TEA; (3) Tmevpg1 recruits WDR5 to induce IFNγ expression; (4) Nron modulates the import–export of NFAT to the nucleus; (5) IFNα1-AS acts as a competing endogenous RNA, releasing IFNα from micro-RNA inhibition. In light red other mechanisms are described for lincRNAs outside the immune system.

Through such a plastic and versatile structure, lncRNAs can exert their functions binding to proteins, other RNAs (98), and probably also DNA, even if there is still little evidence on the existence of RNA:DNA triplex (99, 100). In particular, lncRNAs can act as scaffolds, bridging together different molecules in a coordinated hub, like in the case of NEAT1: a highly abundant lncRNA that controls sequestration of proteins involved in the formation of paraspeckles, nuclear domains associated with mRNA retention and pathologically enriched in influenza and herpes viruses infections (101, 102). LncRNA can also act as guides, recruiting proteins at specific loci: this has been hypothesized in the case of recombination events that mediate genetic diversity in developing lymphocytes as class switch (CS) and V(D)J recombinations that seem to be mediated by sense and antisense transcripts that dictates the locations of combinatorial events (103–105). Again, lncRNAs can act as control devices or riboswitches in response to extracellular stimuli. For example, they can act as decoys, precluding pre-existing interactions such as GAS5 RNA that detach glucocorticoid receptor (GR) from its responsive elements in conditions of growth arrest (106, 107). Nonetheless, the regulatory potential of lncRNAs has been better characterized in the context of the epigenetic regulation of transcription that ultimately defines the cell transcriptome.

THE ROLE OF LONG NON-CODING RNAs IN EPIGENETICS

Histones and DNA modifications together with the tridimensional chromosomes conformation within the nucleus define, at least in part, the epigenetic landscape of the cell. This extremely dynamic context modulates gene expression and dictates the final transcriptional output in response to environmental stimuli. By definition, these modifications are then propagated throughout cell divisions. This process is important in every moment of cell life, but particularly during differentiation. Indeed, every cell within our body harbor the same genome, but every cell acquires a particular phenotype according to intrinsic and extrinsic cues that ultimately defines its epigenome and therefore its fate during differentiation. Epigenetics also defines to what extent this fate can be irreversible or plastic (108–110).

As mentioned before, human lymphocytes are an interesting model system for understanding the basis of cell fate specification and plasticity. Indeed, although traditionally the broad range of effector lymphocytes has been referred to as composed by distinct lineages, it has become increasingly clear that these cells also have notable features of plasticity. Differentiation of naïve cells into specific helper subsets requires the integration of extrinsic cues that converge into cell-intrinsic changes in the epigenetic landscape on the genome (111, 112). The interest within the field has been focused on the regulation of prototypical cytokine genes for each subset such as *Ifng* gene for T_H1 or *Il-4* for T_H2 $CD4^+$ lymphocytes. Much work has been done in both cases to define the complex genetic structure of these loci and the *cis* regulatory elements bound by TFs and chromatin modifiers promoting or repressing their transcription (113–115). The importance of the setting of epigenetic memory at these fundamental loci was underlined also by treatment with DNA methylation inhibitors (116, 117) or histones deacetylases inhibitors (118–120) and by deletion of DNA methyltransferase (121–123), which caused respectively: constitutive production of IFN-γ, enhanced production of both T_H1 and T_H2 prototypic cytokines, and inability to activate the proper pattern of expressed cytokines. The same is true for deletion of components of trithorax group (TrxG) or polycomb repressive complex (PRC) that dictates active or repressive epigenetic marks at fundamental loci for proper T-helper cell differentiation, such as *Il-4*, *Il-5*, *Il-13*, and *Gata3* (124–129). The pattern of chromatin marks is conventional for signature cytokines: active marks are present at prototypical cytokines whereas repressive marks restrain the expression of antagonistic molecules. However, master regulators and other TFs, usually considered as definers of lineage-specific identity, are characterized by bivalent poised domains, in which both active and repressive chromatin marks are present (130, 131). This histone epigenetic status is peculiar also to promoters in embryonic stem cells, where it poises the expression of key developmental genes thus allowing their timely activation in the presence of differentiation signals and concomitantly precluding expression in their absence (132). Indeed, while the expression of master TFs is quite rapid, cell divisions are required for cytokine loci to become accessible or conversely repressed. Indeed, GATA3 and T-bet/STAT proteins initiate the epigenetic changes at IFN-γ and IL-4 loci that follow the initial activation of naïve T cells and differentiation toward T_H1 and T_H2 cell fate (133, 134). These observations imply that T-helper cells harbor both clear-cut and plastic epigenetic marks. Nonetheless, we must consider that even cytokines genes that are clearly defined epigenetically, can be expressed or repressed in unexpected context, as reported in T_H1 cells converted in IL-4-producing cells during strong T_H2-polarizing helminth infections (135) or stable T_H1/T_H2 hybrid cells derived after parasite infections (136). Therefore, other players must be involved to define the degree of plasticity of lymphocytes in response to these ever-changing environmental conditions during differentiation.

Long non-coding regulatory RNAs have been linked to epigenetic control of gene expression since the first studies regarding the already mentioned Xist transcript, involved in X chromosome inactivation in eutherians. Many other lncRNAs have been associated to chromatin or DNA modifiers and even TFs, thanks to specific mechanistic studies or high-throughput screenings (82, 137–139). This interplay can be observed across a broad range of eukaryotic organisms, suggesting that the epigenetic role of lncRNAs is conserved, even if their mere sequence conservation is often limited (as described previously). It seems that lncRNAs could act as scaffolds, physically associating with proteins that modify chromatin either activating or repressing gene expression. Thanks to the already discussed structural properties of RNA, lncRNAs could organize multiple players in spatially and temporally concerted actions (138). Not only: thanks to their ability to base pair with other nucleic acids, they could recruit these modifiers at specific loci, therefore conferring them specificity of action (98). This property has been an unsolved issue, given that chromatin modifiers do not possess intrinsic bias toward consensus sequences, at least in mammals, while in *Drosophila* these "docking sites" are well defined (140, 141). Interestingly, while many of these enzymes lack DNA-binding properties, they instead possess RNA-binding motifs (142–144).

The majority of reported lncRNAs are involved in the repression of gene transcription, in particular by interacting with polycomb group (PcG) proteins. The first examples of a direct interaction with PRC2 are the already mentioned Xist (145) and Kcnq1ot1, expressed only in the mammalian paternal chromosome and involved in the silencing of 8–10 protein-coding genes (146). In both these cases, lncRNAs are strictly required for the enrichment of PRC2-associated proteins and for the trimethylation of the lysine 27 of histone H3 at specific loci. Furthermore, other lncRNAs such as NEAT2 and TUG1 promote relocation of growth-control genes at foci of PcG proteins (called PcG bodies), therefore likely facilitating the concerted repression/activation of the transcription units in response to mitogenic signal (147). Many other protein complexes have been found to interact with lncRNAs, the majority targeting histones, either methylases or demethylases, but other involved in DNA methylation (148). Indeed, lncRNAs can bind proteins part of the TrxG (68) that antagonize PcG-mediated silencing (149). Interestingly, an antisense lncRNA has been recently involved in recruiting a regulator of DNA demethylation at a specific promoter (150). This process remains still largely unknown and it has only recently been associated to active enzymatic reactions, via TET family of methylcytosine dioxygenases (151, 152). Even in this case, one of the unsolved questions has been how locus-specificity can be achieved. Particularly, DNA demethylation is often restricted to few dinucleotides at the TSS. The precise mechanism, though, through which lncRNAs could direct DNA or chromatin modification has never been described. Indeed in all reported examples, correlations have been described between lncRNA-modifiers associations and loss of modification after lncRNA gene silencing.

Long non-coding regulatory RNAs are supposed to confer binding specificity to modifiers and recruiting them either *in cis* or *in trans*. In the first case, lncRNAs could act directly on sites where they are synthesized without needing to leave DNA. The current hypothesis suggests that the 5′ region of the nascent transcript could bind proteins while the 3′ is transcriptionally lagging, being still tethered to chromatin by RNA polymerase (153). This model is particularly intriguing as through this mechanism, lncRNAs could exert an allele-specific effect, like in the well-studied case of Xist. *In trans* regulation is instead achieved when lncRNAs act modulating genes across great distances or even on different chromosomes (154). Regarding this dichotomy, we must underline once again its artificiality. Indeed, chromosomes fold into complex, three-dimensional territories together with specialized subnuclear bodies. Proteins that are part of the transcriptional or splicing machinery and regulators of these processes group at these foci (155, 156). These structures are not static, but on the contrary, large-scale chromosomal repositioning is observed in response to environmental stimuli or during differentiation (157, 158). Subnuclear movements are of key importance in regulating events like transcription and rearrangement that occur at immunoglobulin loci during B lymphocytes development (159). The dynamic folding of the genome into higher order structure encompasses loci belonging to the same chromosome, even hundreds of kilobases apart, or different ones, bringing together regions that are distant if we consider the genome as linear. Therefore, in this context, it is extremely difficult to discern what regulations are *in cis* or *in trans*, especially when they involve long distance interactions. Intriguingly, lincRNAs have been found to regulate the formation of subnuclear structures, such as NEAT1, required for paraspeckles nucleation (101). LncRNAs can also affect directly the three-dimensional organization of chromosomes enhancing the function of proteins involved in looping formation, like the insulator protein CTCF (160). There are also many examples of lncRNAs involved in three-dimensional local chromatin looping that brings together the ncRNA gene with the region that it regulates within the same chromosome (68, 161). Recently, a lincRNA called Firre has been shown to recruit specific gene loci located on different chromosomes, acting as a docking station for organizing *trans*-chromosomal associations. Consistently, genetic deletion of Firre leads to a loss of proximity of several *trans*-interactions (162). A peculiar type of lncRNA has been described that is transcribed from enhancer regions (eRNAs). Classic enhancer elements therefore likely act through transcription of these lncRNAs that upregulate expression at promoters via the recruitment of Mediator complex (163, 164). Finally, there is increasing evidence that even promoters could be transcribed (165), producing lncRNAs probably involved in the enhancer–promoter loop that was hypothesized years ago but never fully resolved (166).

LONG NON-CODING RNAs IN THE ADAPTIVE IMMUNE SYSTEM

The adaptive immune system is an extraordinary context for the study of the role of lincRNAs in differentiation. Indeed, upon antigen stimulation, naïve $CD4^+$ T cells differentiate into distinct T-helper subsets that were traditionally considered lineages and defined by a prototypic set of expressed cytokines and master TFs. Recently, this relative simple scenario, although useful, has been subjected to debate. $CD4^+$ T cells demonstrated to exhibit substantial plasticity and it has become increasingly clear that they can change the pattern of cytokines and TFs according to the milieu they encounter through their life (167, 168). Not only, in some cases, they can concomitantly express other cytokines and TFs together with their prototypical set. Best examples include IL-10, once thought to specifically identify T_H2 and now known to be produced also by T_H1, T_{reg}, and T_H17 cells (169) and IFN-γ, the classic T_H1 cytokine, frequently released by T_H17 cells simultaneously with IL-17 (170, 171). Regarding master TFs, T_{regs} can express *Foxp3* (their prototypical TF), but also *RORγt* (T_H17 TF) and *Runx3* (172–174); similarly T_{FH} cells can differentiate from FOXP3 positive cells also expressing *Bcl6* (their specific TF) (175, 176). In this context, lncRNAs have a fundamental role in governing flexibility and plasticity or maintenance of cell identity, together with lineage-specific TFs and other ncRNAs. In particular, what is emerging from the literature is that ncRNAs typically act as fine-tuners of fate choices and this seems to be true not only in the immune system. Nonetheless, in the case of $CD4^+$ T-cell subsets that are specified but not fully determined, subtle changes in extrinsic signals can reverberate through responsive ncRNAs inducing changes that alter cell phenotype (38, 177, 178). Usually, the stability of lineage identity is achieved through the implementation and inheritance of epigenetic modification, but as mentioned before, lncRNAs can act directly on histone and DNA modifiers redefining this context.

Conversely, lncRNAs can also buffer this situation in other conditions, acting as maintainers of cell identity. In the cellular system, lncRNAs can be regarded as minor nodes in a huge interconnected network (179), as they usually interact with few other players. This condition allows them to be more flexible and sensitive to variations without disrupting the whole network integrity (180). This is true both over a very short period, as cells can easily and rapidly adapt to environment, and also over long evolutionary periods, as lncRNAs are among the fastest evolving sequences in the genome (95, 181–183). Conversely, master transcription regulators can be considered highly connected hubs, which confer robustness to the network. Indeed, very few protein-coding genes have been lost from worms to human and mutations are most often pathological (184, 185).

Several single-case or genome-wide studies on lncRNAs in the murine adaptive immune system or cell lines are now available in the literature, whereas only few studies have been conducted until now in the human context. The number of studies that unveiled the function and mechanism of a specific lncRNA is so small that can be counted on one hand (**Table 3**).

The importance of the studies in the human immune system is underlined by the fact that the differences between experimental animal models and human are still subject of debate in terms of immunologic responses (199–201). Moreover, there are increasing evidences that ncRNAs are poorly conserved between animal models and human (202, 203). In particular, lncRNAs are really fast-evolving elements as demonstrated by the fact that over 80% of the human lncRNAs that arose in the primate lineage, only 3% are

Table 3 | Studies on lncRNAs in the adaptive immune system.

Sample	LncRNAs	Function
Granulocytes, monocytes, NK, B, naïve $CD8^+$ and $CD4^+$, memory human T cells; *in vitro* polarized precursors T-helper, T_H0, T_H1, and T_H2 human cells	240 lncRNAs associated with autoimmune disease (AID) loci (RNA-seq)	Analysis of the expression profile of the AID-associated lncRNAs (186)
$CD4^-CD8^-$, $CD4^+CD8^+$, $CD4^+CD8^-$, activated $CD4^+$ mouse T cells	31423 lncRNAs (lncRNA microarray)	Expression analysis and prediction of function (187)
17 T-cell leukemia cell lines	Thy-ncR1 (expression profiling of 10 thymus-specific ncRNA)	Enriched in human immature cells; acts as a cytoplasmic riboregulator that reduces the level of MFAP4 mRNA (188)
Naïve, memory, activated, non-activated mouse $CD8^+$ T cells	Over 1000 mouse and human lncRNAs (microarray)	Expression and conservation analysis (37)
$CD4^-CD8^-$, $CD4^+CD8^+$, $CD4^+$, $CD8^+$ mouse thymic T cells, and thymus-derived T_{reg} cells. *In vitro* differentiated T_H1, T_H2, T_H17, and induced T_{reg}	1524 lincRNA genes (RNA-seq); LincR-Ccr2-5′AS	Expression analysis and ChIP-seq data analysis to identify lincRNA genes and possible regulators. LincR-Ccr2-5′AS is T_H2-specific and it reduces the expression of *Ccr1*, *Ccr2*, *Ccr3*, and *Ccr5*. It contributes to the migration of T_H2 cells (189)
Infected Namalwa B lymphocytes	IFNA1-AS	Cytoplasmic post-transcriptional stabilization of IFN-α1 RNA masking a miRNA-binding site (190)
Jurkat cells, primary lymphomas, lymphoma cell lines, $CD19^+$ B cells	Saf/FAS-AS1	Regulates the alternative splicing of Fas which is impaired in non-Hodgkin's lymphomas associated with poor prognosis (191, 192)
Activated human $CD4^+$ T cells	*BIC* RNA (EST library analysis)	Proto-oncogene, induced upon activation, sensitive to immunosuppressive drugs (193)
Jurkat cells	NRON (shRNA knock-down screening)	Regulates NFAT subcellular localization as part of an RNA–protein complex (84)
CEM-C7 CKM1, jurkat JKM1, human primary lymphocytes	GAS5	Necessary and sufficient for growth arrest. Acts competing from GREs (71, 106)
Human $CD4^+$, $CD8^+$ cells, PBMC	*NTT*	Unknown, it shows a similar expression pattern to *IFNγR* (194)
Thymocytes	TEA	Instruct the activity of Jα promoters and recombination (103, 195)
Human T_H1 cells	NeST/Tmevpg1/IFNG-AS1	Dependent on STAT4, T-bet, and NFκB. Contributes to *Ifng* expression by binding WDR5 and alter H3K4me3 (196, 197)
Human primary $CD4^+$ and $CD8^+$ T cells, primary and polarized (from $CD4^+$ and $CD8^+$ T) $CD4^+$ CM, T_H1, T_H2, T_H17, and T_{reg} cells; neutrophils, basophils, $CD8^+$ CM, B cells	*GATA3-AS1*	Specifically expressed in T_H2 cells (198)

conserved across tetrapods and most mammalian lncRNAs lack known orthologs outside vertebrates (97). In detail, even between mouse and human, lncRNAs are poorly conserved (204–206). Despite their rapid evolution, lncRNAs are selected more than neutral sequences and in particular more than intergenic regions, but significantly less than mRNAs (96, 97, 207). It must be underlined that the conservation rate reported could be overestimated: substitution rates are derived from whole-genome alignment and based on the assumption that even segment of homologies imply that that segment belongs to the same RNA class, but this is not necessarily the case. Indeed, it could be that in another genome context a specific lncRNA gene segment is transcribed and processed as part of a protein-coding RNA (208). A striking example is Hotair that is involved in the regulation of the highly conserved cluster of *Hox* genes (68). The human lincRNA is conserved in the mouse genome (209), nonetheless only the 3′ region is effectively part of the murine homolog (183). The importance of studying lincRNAs specifically within the human immune system derives from these considerations, but this field is still poorly investigated. The majority of the studies focused on the innate immune system (210–212) or analyzed pathological situations, such as cancer-related lncRNAs (192, 213) or responses to specific infections (102, 214–216), mostly in mice. The first functional study that focused on the adaptive immune system, and in particular on T_H1 and T_H2 lymphocytes, involved a lincRNA, Tmevpg1, that is selectively expressed in T_H1 cells via STAT4 and T-bet, both in mouse and human. It participates in the induction of IFN-γ expression strictly in response to T_H1 differentiation program and not in other cellular contexts. These results highlight once again the complexity of gene expression regulatory network and the specificity of action of lincRNAs (196). Another paper described a lincRNA, GATA3-AS1, specifically expressed in primary T_H2 cells and hypothesized its co-regulation with GATA3 (198). GAS5, expressed in human T lymphocytes, is degraded in optimal growth conditions, but it accumulates contributing to growth arrest in starving conditions (107). In this situation, it competes with GRs DNA-binding sequences, suppressing GR-mediated transcription (106). Broader studies have been performed on the $CD8^+$ T cell transcriptome (37), and recently on $CD4^+$ T lymphocytes (189), but still on mice models. In B cells, chromatin remodeling associated with V(D)J recombination has been potentially linked to a widespread antisense intergenic transcription that occurs in the variable (V) region of the immunoglobulin heavy chain (Igh) locus (104, 105). So far, no studies have been published that performed a deep transcriptomic analysis on human primary lymphocytes from healthy donors, identifying lncRNAs fundamental for differentiation processes. These few examples are just clues of the importance that lincRNA could have for the proper function also of the human immune system and prompt to a deeper analysis of their role in this particularly intriguing context.

LONG NON-CODING RNAs AS EPIGENETIC MODULATORS IN LYMPHOCYTE DIFFERENTIATION

Traditionally, the secretion of IFN-γ and TNF-α characterizes T_H1 lymphocytes, whereas IL-4, IL-5, and IL-13 are considered prototypic cytokines secreted by T_H2 cells. According to this classic paradigm, these differences underline the different functions exerted by these lymphocytes: T_H1 are considered as important to eliminate intracellular bacteria and viruses, whereas T_H2 to resist parasitic infections (217). The advantage of solid *in vitro* differentiation protocols allowed a deep understanding of the genetic mechanisms governing these cells. Since the discovery of this dichotomy, other cell subsets have been identified, but this T_H1/T_H2 paradigm was undoubtedly useful. Therefore, it is not a case that among the few lncRNAs identified in the immune system, many of those functionally characterized have been described in these two cell subsets. Nevertheless as mentioned before, just one lincRNA, Tmevpg1 (also known as NeST or IFNG-AS1) has been characterized in deep. Tmevpg1 is located proximal to IFN-γ gene both in mice and humans, antisense and convergently transcribed respect to the neighboring gene and plays a role in chromatin remodeling. This transcript is a T_H1-specific lincRNA: it requires STAT4 and T-bet for being transcribed and is also bound by CTCF and cohesin during lineage-specific induction (196). Therefore, Tmevpg1 is directly dependent on the activation of a T_H1-polarizing transcriptional program, in which the presence of IL-12 leads to the activation of the JAK/STAT pathway via STAT4 (and STAT1) that induces the expression of T-bet. Interestingly, *Tmevpg1* gene harbor sequences regulated by histone acetylation and DNase I hypersensitive sites found in T_H1 but not T_H2 cells (218, 219). Tmevpg1, in its turn, plays a direct part in defining the proper T_H1 cytokine expression pattern, influencing *Ifng* transcription in the presence of T-bet (196), via H3K4 trimethylation by WDR5 binding in mice models (197).

Given the increasing number of lncRNAs described in different cellular contexts and the high number of specific lincRNAs expressed in the different lymphocytes subsets identified with the aforementioned RNA-seq analysis, many more lincRNAs will likely be characterized in the future with a relevant function in the human immune system. A major limitation, though, in the studies on lncRNAs is that there is little biological knowledge on the biochemical or molecular function of lncRNA genes. Compared to classical protein-coding gene studies, hints on their functions cannot be gained simply by the analysis of their primary sequence and application of computational methods to infer lncRNA function are also still in their infancy. As lincRNAs have been reported to influence the expression of neighboring genes (25, 26, 28, 39), one possible approach to investigate their putative function is to focus on lymphocyte lincRNAs proximal to protein-coding genes involved in key cell-functions.

Through this approach, we identified a T_H1-specific lincRNA that localized ~140 kb upstream to *MAF* that was therefore called linc-MAF-4. MAF is a TF involved in T_H2 differentiation and required for the efficient secretion of IL-4 by T_H17 and the proper development of T_{FH} cells (220–222). Intriguingly, the expression of *linc-MAF-4* is negatively correlated with respect to the expression of *MAF*: linc-MAF-4 expression is high and specific in T_H1 lymphocytes, where *MAF* is lowly expressed whereas in T_H2 cells the expression of *linc-MAF-4* is extremely low and *MAF* is highly expressed. Coherently, linc-MAF-4 knock-down in naïve $CD4^+$ T cells increased the expression of *MAF* and interestingly induced a more general skewing of the whole transcriptomic profile of these cells toward a T_H2-like fate (63). The regulation exerted by linc-MAF-4 on *MAF* gene was analyzed in more detail and this lincRNA

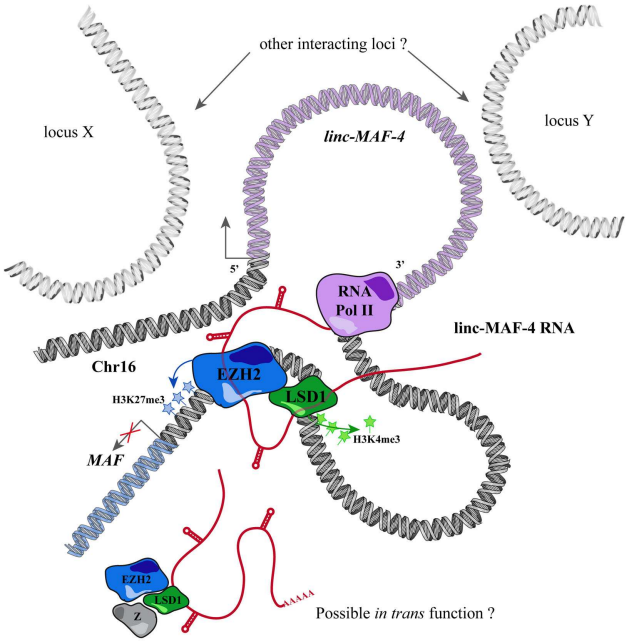

FIGURE 2 | Mechanism of action proposed for linc-MAF-4.

proved to modulate *MAF* expression *in cis*, as hypothesized by expression analysis. Linc-MAF-4 exerts this regulation by exploiting a chromatin loop that brings its genomic region close to the promoter of *MAF* gene. Indeed, the chromatin organization of this region allows linc-MAF-4 transcript to recruit chromatin remodelers that inhibit *MAF* transcription. In particular, linc-MAF-4 was found to associate with EZH2, key enzymatic subunit of the PRC2 complex, and LSD1. These proteins methylate H3K27 and demethylate H3K4, respectively: two histones modifications that code for transcriptional repression. (63). A similar mechanism was described for other lincRNAs, such as HOTAIR and MEG3 (154, 223) but never before for other lncRNAs expressed in the adaptive immune system (**Figure 2**).

Changes of lincRNAs expression during naïve to memory $CD8^+$ T-cell differentiation (37) and during naïve $CD4^+$ T cells differentiation into distinct helper T-cell lineages (189) have been described in the mouse immune system. linc-MAF-4, though, is, to our knowledge, the first example of a lincRNA playing a role in the proper differentiation of human T_H1 cells, suggesting that, besides cytokines and TFs, lncRNAs take part in the T_H1 differentiation program as already shown in many other cell types. At this point, an obvious question arises: to what extent are these cells plastic? These findings are evidences that it is possible to redirect the differentiation path of naïve $CD4^+$ T cells acting on their lincRNA content. Nevertheless, could it be possible to modulate already committed cells? We would expect that the mere down-regulation of a lincRNA would not be sufficient nor a lincRNA knock-out: as mentioned before, lincRNAs are minor nodes in a huge interconnected network composed by feedback mechanisms and epigenetic marks that act stabilizing a pre-existent differentiation status. However, a modulation in lincRNA content may be sufficient to make these cells more responsive to environmental cues that could overcome stabilizing forces, inducing a sort of trans-differentiation event. Functional characterization of other lncRNAs is required to address this crucial issue and to assess the extent of their contribution to cell differentiation and to the maintenance of cell identity in human lymphocytes. Based on what we discussed so far on lncRNA functions and cell-specificity, we believe that future studies will show how these molecules could be capitalized as new molecular targets for the development of novel and highly specific therapies for diseases, such as autoimmunity, immunodeficiencies, allergy, and cancer.

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Cellular plasticity of CD4+ T cells in the intestine

Verena Brucklacher-Waldert†, Edward J. Carr†, Michelle A. Linterman and Marc Veldhoen*

Laboratory for Lymphocyte Signalling and Development, The Babraham Institute, Cambridge, UK

Edited by:
Dragana Jankovic, National Institutes of Health, USA

Reviewed by:
John J. Miles, Queensland Institute of Medical Research, Australia
Sid P. Kerkar, National Institutes of Health, USA

***Correspondence:**
Marc Veldhoen, Laboratory for Lymphocyte Signalling and Development, The Babraham Institute, Babraham Research Campus, Cambridge CB22 3AT, UK
e-mail: marc.veldhoen@babraham.ac.uk

†Verena Brucklacher-Waldert and Edward J. Carr have contributed equally to this work.

Barrier sites such as the gastrointestinal tract are in constant contact with the environment, which contains both beneficial and harmful components. The immune system at the epithelia must make the distinction between these components to balance tolerance, protection, and immunopathology. This is achieved via multifaceted immune recognition, highly organized lymphoid structures, and the interaction of many types of immune cells. The adaptive immune response in the gut is orchestrated by $CD4^+$ helper T (Th) cells, which are integral to gut immunity. In recent years, it has become apparent that the functional identity of these Th cells is not as fixed as initially thought. Plasticity in differentiated T cell subsets has now been firmly established, in both health and disease. The gut, in particular, utilizes $CD4^+$ T cell plasticity to mold $CD4^+$ T cell phenotypes to maintain its finely poised balance of tolerance and inflammation and to encourage biodiversity within the enteric microbiome. In this review, we will discuss intestinal helper T cell plasticity and our current understanding of its mechanisms, including our growing knowledge of an evolutionarily ancient symbiosis between microbiota and malleable $CD4^+$ T cell effectors.

Keywords: T cells, plasticity, intestines, Th1 cells, Th17 cell

INTRODUCTION

The adult human gastrointestinal tract is the largest surface area of the body that contacts with the environment, covering 200–300 m^2 (1). This intestinal surface is constantly exposed to a diverse range of foreign antigens originating from microorganisms (both commensals and pathogens) and food antigens from the diet (2). Commensal microorganisms play an essential role in extracting nutrients from food that are otherwise inaccessible to the host [such as the metabolism of vitamin K by *E. coli* (3)], they are required for the development of the host's immune system and for the prevention of colonization of the gastrointestinal tract by pathogens. Mucosal pathogens, including viruses, fungi, parasites, and bacteria, can cause pathology either by local effects after mucosal colonization – such as inducing local inflammation or secreting toxins – or, through systemic infection after breeching mucosa. Microorganism-derived antigens, food-derived antigens, and airborne particles, can be potential immunogens. An inappropriate response to these immunogens at the mucosal surface can be detrimental, leading to local or systemic pathology that result in acute or chronic inflammation. Therefore, it is essential that the myriad of antigens present at the intestinal surface is dealt with appropriately to minimize potential danger and maximize host benefit. This protection is achieved by a flexible, multilayered system of physical, and immunological barriers within the gastrointestinal tract.

A central part of the complex host defense system is gut-associated lymphoid tissue (GALT). GALT is a system of highly organized immune structures strategically placed along the entire gastrointestinal tract, containing specialized micro-environments where gut-derived antigens are presented by professional antigen presenting cells (APCs) to lymphocytes [reviewed recently in Ref. (4)]. The broad antigenic sampling within the GALT facilitates the interaction between rare antigen-specific B and T cells leading to the initiation of an appropriate adaptive immune response (5). $CD4^+$ T cells are critical players in the adaptive immune response within the GALT. Naïve T cells egress from the thymus as immature T cells with a broad range of T cell receptors (TCRs) and can be activated in the periphery following encounter with their specific antigen. T cell activation is initiated by ligation of the TCR by peptide–MHC class II complex in conjunction with co-stimulatory signals. During T cell priming, cytokine receptor ligation can skew activated T cells into a particular effector cell type (6). These cell types are commonly referred to lineages or subsets, with each being identified by selected expression of characteristic transcription factors and effector molecules (7). With the exception of thymically derived regulatory T cells (Tregs) [reviewed in Ref. (8)], thymic emigrants lack any predisposition to make effector molecules associated with a particular $CD4^+$ T cell subset, and require signals in the periphery to skew their differentiation into a particular cell subset. Present understanding of T cell lineage commitment is dominated by single fate model, a process whereby a naive T cell differentiates along a terminal fixed expression program, in response to signals at the time of antigen encounter. However, this view has needed revision in light of findings from many groups, which together demonstrate that $CD4^+$ T cell subset fate is not a permanent attribute, but rather a flexible, plastic, feature that can be modified to suit the requirements of the immune system at a particular point in space and time. Thus, the new paradigm of T cell differentiation encompasses the ability of $CD4^+$ T cells to change between expression programs traditionally thought to be mutually exclusive terminal states of differentiation (9). This feature has been reported *in vivo* in several experimental systems, and intriguingly, is a prominent feature of $CD4^+$ T cell biology within the GALT.

The detailed mechanisms underlying T cell plasticity within the GALT remains to be defined, but several factors that facilitate its occurrence have been proposed and can be divided into extrinsic and intrinsic pathways. In this review, we will summarize the recent literature on CD4$^+$ T cell plasticity in the gut, highlight possible underlying mechanisms and discuss its potential benefits for intestinal homeostasis and health.

CD4$^+$ T CELL DIFFERENTIATION IN THE GALT

The GALT contains one of the largest lymphoid cell population found anywhere in the body. GALT is distributed along the intestinal tract and is separated from the luminal content, containing about 100 trillion microorganisms (10) and many dietary products, by a single epithelial layer covered with an intricate network of glycoproteins; the mucous layers. The GALT provides three functions: provides antigenic samples from throughout the GI tract; optimizes the opportunities for naïve lymphocytes to encounter antigen, and finally supports the activated lymphocyte and its initial differentiation. A network of highly organized lymphoid structures comprise the GALT (**Figure 1**) – including mesenteric lymph nodes (mLNs), Peyer's patches (PPs), isolated lymphoid follicles (ILFs), cryptopatches (CPs), and fat-associated lymphoid tissues – as well as the loose connective tissue of the lamina propria (LP). Despite the numerous types of GALT, the organization of all GALT lymphoid tissues shares a basic cellular architecture that facilitates the interaction of APCs with lymphocytes and their subsequent activation and differentiation. However, the GALT tissues differ from each other both in function and in the physical distance to the intestinal lumen; CP and ILFs are located directly underneath the epithelial layer, PPs are further from the lumen and most distant are mLNs and fat-associated lymphoid tissues. The specialized functions of each the GALT tissues have been recently reviewed in Ref. (4), and therefore will not be discussed in detail in this review.

Naïve L-selectin-expressing CD4$^+$ T cells migrate from the blood through high endothelial venules (HEVs) to the PPs, CP, ILFs, and mLNs. In these inductive sites of the adaptive immune system, they encounter their cognate antigen presented by APCs on MHC class II. Naïve T cells that did not encounter their cognate antigen leave via efferent lymphatic vessels into the systemic circulation to continue their search for their cognate antigen. In PPs, CP, and ILFs, dendritic cells (DCs) receive antigens transported from the mucosal surface by specialized epithelial cells overlying these lymphoid structures, the microfold (M) cells, and Goblet cells [reviewed in Ref. (4)]. mLNs receive antigens via afferent lymphatics that are connected with the PPs, while fat-associated lymphoid tissues obtain antigen by lymphatic drainage directly from the intestine and from the peritoneal cavity (4).

After activation in the intestine CD4$^+$ T cells proliferate, shed L-selectin from their surface, increase surface expression of gut-specific adhesion molecules and differentiate into an effector T cell, of a particular subset. Antigen-experienced effector T cells usually leave via efferent lymphatic vessels and home to effector tissues such as the intestine after re-circulation in the blood, but

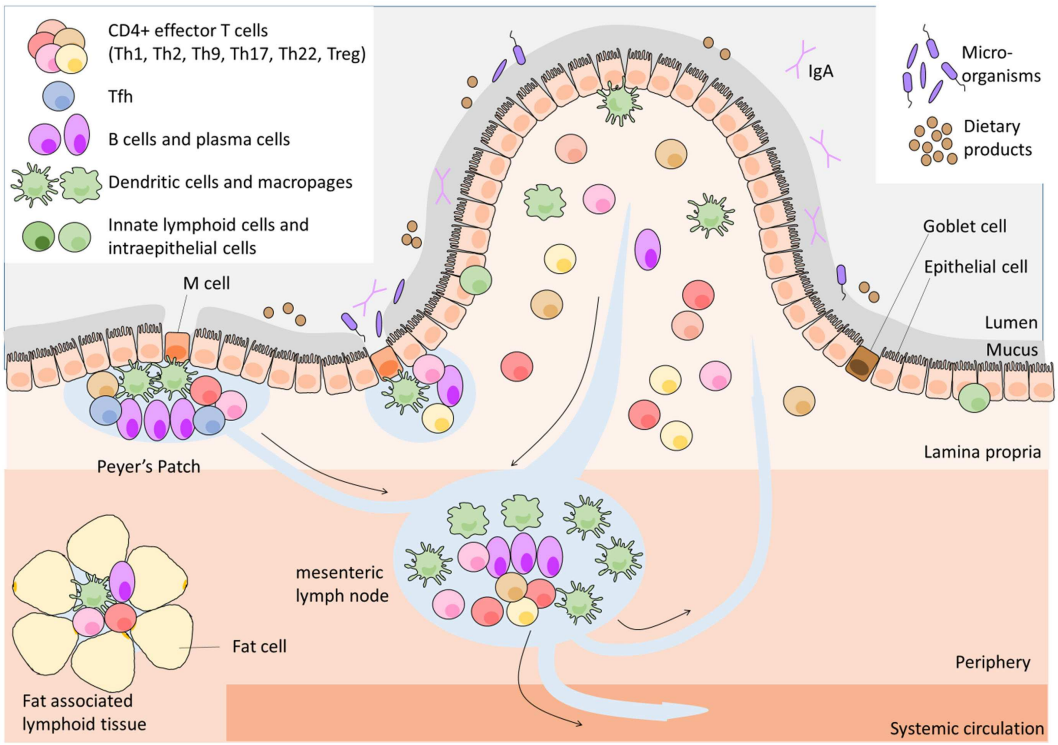

FIGURE 1 | Architecture of gut-associated lymphoid tissue.

some remain in the lymphoid organs to perform their specialized effector functions (11). Upon arriving in the intestine, effector T cells are likely to re-encounter their specific antigen presented by a diverse range of cells such as macrophages, B cells various types of DCs, and stromal and epithelial cells. This second antigen-specific interaction will trigger the T cells to execute their effector function.

It is the nature of antigen presentation and the surrounding micro-environment that largely seems to determine the outcome of $CD4^+$ T cell differentiation and the subsequent spectrum of T cell identities leaving the lymphoid structures (6). The $CD4^+$ T cell subsets currently recognized are: T helper cells of type-1 (Th1), Th2, Th9, Th17, Th22, Tregs, and follicular helper T cells (Tfh). Each subset is characterized by the potential to produce a defined set of mediators such as cytokines and chemokines, respond to particular stimuli via the expression of selected cytokine and chemokine receptors, largely driven by so called "master regulator" transcription factors (summarized in **Table 1**).

$CD4^+$ T CELL PLASTICITY

Plasticity of effector $CD4^+$ T cells has been increasingly recognized in recent years. Initially, there were single reports of observations of particular subsets behaving "non-typically," by the expression of transcription factors, cytokines, or both, of another subset. Together these various reports support a more nuanced view of Tfh differentiation, in place of the monolithic view of distinct, non-interchangeable lineages (12). T helper cells are able to acquire a mixed phenotype or to switch entirely to the transcription and cytokine profile of another lineage (**Table 2**). These hybrid cells or ex-Th lineage cells may either directly differentiate from naïve $CD4^+$ T cells or arise during secondary immunological challenge. Current data suggest that, within the gut, particular transitions between $CD4^+$ T helper cell subsets occur and are important for maintaining gastrointestinal homeostasis, as outlined below.

Th1/Th2 CELL PLASTICITY

T helper cells of type-1 cells are an abundant effector population in the gut responsible for protection against invading intracellular bacteria and viruses (31). In contrast, Th2 cells are absent in the intestine of laboratory mice and humans under healthy conditions (31, 32); not surprising in view of their role in coordinating host responses to helminths and the absence of these organisms in a healthy intestinal flora (31). Identification of Th1 and Th2 as distinct cell types was the first, time where $CD4^+$ T cell subsets were divided on the basis of phenotypic differences *in vitro* (33). It is therefore interesting that culture of T cells under mixed Th1 and Th2 conditions results in a continuum of cytokine expression, with some single positive $IFN\gamma^+$ or $IL\text{-}4^+$ and double positive cells, correlating with expression levels of Tbet and GATA-3 (34), the respective master transcription factors for the Th1 and Th2 subsets, demonstrating these cell fates are not a distinct as originally thought. Importantly, this finding is consistent with *in vivo* observations; during infections with *Heligmosomoides polygyrus*, a parasite that triggers a strong Th2 response, Th1/Th2 hybrid cells were observed (13). In the intestine, mLNs and spleen, these Th1/Th2 hybrid cells express simultaneously the Tbet and GATA-3 and not transcription factors of other subsets [retinoic acid (RA) receptor-related orphan nuclear receptor (ROR)γt or Bcl-6]. In addition, they express the Th1 cell-associated molecules CXCR3 and IFNγ concordantly with the Th2 cell-associated IL-33R, IL-4, IL-13, and IL-5. Furthermore, Th2 cells *in vivo* can express Tbet and IFNγ upon infection with lymphocytic choriomeningitis virus (LCMV), a potent Th1 skewing pathogen. These Th1/Th2 hybrid cells represent a stable phenotype, where attempts at reprograming by stimulation under Th1 or Th2 polarizing conditions result in quantitative changes of Th1/Th2 cytokine production without fully extinguishing either. Upon transfer into wild-type mice, around 20% of LCMV-specific Th1/Th2 cells maintained their expression of GATA-3, IL-4, and IL-13 alongside Tbet and IFNγ

Table 1 | Effector $CD4^+$ T cells in the gut.

Cell type	Differentiation signals	Transcription factor (master transcription factor)	Cell surface and cytokine markers	Location in the gut under homeostatic conditions	Function in the gut
Th1	IL-12, IFNγ	*Tbet*, STAT1, STAT4, and Runx3	CXCR3, IFNγ	mLN, PP, CP, ILF, and LP	Responding to intracellular pathogens and assisting with viral infections
Th2	IL-4, IL-2	*GATA-3*, STAT6, and STAT5	IL-33R, IL-4, IL-13, and IL-5	Virtually absent	Responding to helminths
Th9	TGFβ, IL-4	PU.1	IL-9	Exact location to be determined	Involved in tumor immunology
Th17	IL-6, TGFβ, IL-1β, and (IL-23, IL-21)	*RORγt*, AhR, STAT3, Batf, Runx1, and RORα	CD161, IL-17A, IL-17F, and GM-CSF	mLN, PP, CP, ILF, and LP	Protect the host from infectious assault at mucosal site
Th22	IL-6, IL-13, and TNFα	AhR	CCR4, CCR6, CCR10, and IL-22	Exact location to be determined	Wound repair and induction of antimicrobials
Tfh	IL-6, IL-21	*Bcl-6*, Ascl2	CXCR5, PD-1, and IL-21	PP, ILF?	Induce IgA production by GC B cells to maintain a healthy microbiota
iTreg	TGFβ, IL-2	*Foxp3*, STAT5	IL-10, TGFβ	mLN, PP, CP, ILF, and LP	Suppressor capacity, keep homeostatic balance in gut

Table 2 | CD4$^+$ T cell plasticity in the gut.

Plasticity in the intestine	Capacity	Features of hybrid cell/ex-Th lineage cell	Origin	Developmental mechanism	Function in the gut	References
Th1/Th2	Th1/Th2 hybrid cells	Tbet$^+$ CXCR3$^+$ IFNg$^+$ GATA-3$^+$ IL-33R$^+$ IL-4$^+$ IL-13$^+$ IL-15$^+$	Naïve T cells	*H. polygyrus* infection → transcription factor balance	Gut function unknown; ↓ delayed-type hypersensitivity ↓ allergic airway inflammation	(13)
Th17/Th1	Th17/Th1 hybrid cells	RORγt$^+$ Tbet$^+$ IL-17$^+$ IFNγ$^+$ CD161$^+$ (only in humans) β7$^+$ CCR6$^+$	Naive T cells	Cytokine-induced upregulation of Tbet	Associated with Crohn's disease	(14–21)
	ex-Th17^{Th1} cells	Tbet$^+$ RORγt$^-$ IL-17$^-$ IFNγ$^+$ β7$^+$ CCR6$^+$	Committed Th17 cells			
Th17/Th2	Th17/Th2	RORγt$^+$ GATA-3$^+$ IL-4$^+$ GM-CSF$^+$ IL-17$^+$	Not investigated	CD4-dependent Bcl11b-knock down or IL-4 treatment of EAE mice	↓ EAE (by redirecting lymphocytes to the gut)	(22)
Th17-to-Treg	Co-expression of Treg and Th17 markers not shown		Committed Th17 cells	PPARγ activation that promotes iTreg induction	↓ T cell transferred colitis	(23)
Treg-to-Th17	Treg/Th17 hybrid cells	Foxp3$^+$ RORγt$^+$ IL-17$^+$ β7$^+$ CD103$^+$ suppressive	Committed Treg cells	Micro-environmental cues in PPs of Crohn's disease patients	↓ Crohn's disease	(24, 25)
Th17/Tfh	ex-Th17^{Tfh}	RORγt$^-$ IL-17$^-$ Bcl-6$^+$ CXCR5$^+$ PD-1$^+$ IL-21$^+$	Committed Th17 cells	Not IL-23	Required for IgA-producing B cells	(26)
Treg/Tfh	ex-Treg^{Tfh} cells	Foxp3$^-$ Bcl-6$^+$, IL-21$^+$, CXCR5$^+$, CD40L$^+$, ICOS$^+$, PD-1$^+$, and CD28$^+$	Committed Treg cells	Micro-environment of PPs. Requirement for B cells and CD40:CD40L interaction. Involvement of IL-6 and autocrine IL-21	Induced GC formation in PPs and IgA-producing cells in the gut	(27, 28)
CD4$^+$/cytotoxic CD4$^+$ T cells	CD4$^+$ CTLs	CD4$^+$ CD8αα$^+$Runx3$^+$ ThPOK$^-$ granzyme B$^+$	CD4$^+$ T cells	Continuous antigenic stimulation, IL-15, non-pathogenic microorganisms, TGFβ, RA	↓ Colitis	(29, 30)

expression many months after systemic infection with LCMV. Earlier work from the same group has shown that the development of LCMV-specific Th1/Th2 hybrid cells originating from antigen-experienced Th2 cells required TCR engagement, not just a "Th1 milieu" provided by LCMV challenge and are stable *in vivo* for months (35).

Functionally, the Th1/Th2 hybrid cells have the capacity to elicit Th1 and Th2 cell responses but with a decreased Th-specific potency compared their single identity counterpart. Mice that received *in vitro* derived Th1/Th2 cells before the induction of type-1 inflammation showed signs for delayed-type hypersensitivity but not as strong as after a transfer of pure Th1 cells. Similarly, the transfer of Th1/Th2 cells reduced the signs for induced allergic airway inflammation compared to a Th2 cell transfer (13).

Th17/Th1 CELL PLASTICITY

In health, Th17 cells preferentially home to small intestinal LP (26) and are important for intestinal homeostasis [reviewed in Ref. (36)]. Th17 cells are characterized by the expression of IL-17A, IL-17F, and IL-22 and the lineage-defining transcription factor RORγt (37–39), which acts in co-operation with other transcription factors, including aryl hydrocarbon receptor (AhR) and RORα (40, 41). Shortly after the first descriptions of Th17 cells, cells making both IL-17 and IFNγ, so called double producers, were noted. In the intestine of patients with Crohn's disease (CD), IL-17A IFNγ-double positive $CD4^+$ T cells co-expressing RORγt and Tbet have been found (14), sometimes with frequencies above those of single positive T cells (15). Cosmi et al. characterized these double producers further and found that virtually all $IL\text{-}17^+IFN\gamma^+CD4^+$ T cells, as well as Th17 cells expressed the lectin-like surface molecule CD161 (15). This led them to conclude that human Th17 and Th17/Th1 cells exclusively originate from an NKT-like $CD161^+CD4^+$ T cell precursor. Furthermore, Kleinschek et al. showed that $CD161^+CD4^+$ T cells preferentially home to the gut due to their high expression levels of CCR6 and integrin β7. They confirmed that these cells can be induced to produce IFNγ in addition to IL-17 (16). In mice, colitis studies revealed not only the presence of Th1/Th17 hybrid cells (17–19) but also that Th17 cells, initially unable to produce IFNγ, abolish IL-17 production completely and switch on IFNγ (ex-$Th17^{Th1}$) (18, 20, 42).

Hirota et al. used an IL-17-fate reporter mouse that permanently marks IL-17-producing cells to show that these ex-$Th17^{Th1}$ cells undergo a near complete switch from Th17 cell to Th1 cell, except for the maintenance of IL-1R and AhR expression as remnants of their former identity (26). Since Th1 cells are not known for the expression of the IL-1R, ex-$Th17^{Th1}$ cells are unique within the Th1 lineage in the ability to respond to the acute phase reactant IL-1. AhR expression has been shown to increase Th17 cell activity and particularly drive the expression of IL-22 (40). It is currently not known what the role of AhR is in ex-$Th17^{Th1}$ cells, however, it is tempting to speculate that there is a role in coordinating IL-22 production at barrier sites such as the intestine, where IL-22R is expressed by epithelial cells (43). IL-22 deficiency worsens the colitis induced by either dextran sodium sulfate or by T cell transfer (44), and results in reduced secretion of antimicrobial agents important for maintaining epithelial health (45, 46). Interestingly, AhR has been shown essential for the long-term maintenance of lymphoid cells, such as innate lymphoid cells (ILC) type 3, intraepithelial lymphocytes (IELs), and tissue resident cells, at epithelial barrier sites (47–49). This suggests that ex-$Th17^{Th1}$ cells may be maintained in the intestine providing long-term protection or upholding aberrant immunity, in part through IL-22 signaling, with the help of AhR ligands.

Because the frequency and number of IL-17/IFNγ double producers are increased in the gut of CD patients (14), they have been implicated in disease pathogenesis. However, it is unknown if the presence of Th17/Th1 hybrid cells in CD patients are a cause or a consequence of the disease. These hybrid cells could develop as a bystander product of inflammation, or might drive the initial inflammatory response. In colitis models, the pathogenicity of Th1/Th17 hybrid cells and ex-Th17 cells is linked with Tbet. However, the role for Tbet in the pathogenicity of Th17 cells remains controversial (50–53) and switching to Th17/Th1 or $IFN\gamma^+$ex-Th17 might involve Runx family members in co-operation with Tbet (54).

Th17/Th2 CELL PLASTICITY

Naïve T cell activation *in vitro* in the presence of IL-4 inhibits RORγt expression and IL-17 production (55), which makes the existence of T cells with a hybrid phenotype of Th17/Th2 cells seem unlikely. However, *in vivo* studies identified $CD4^+$ T cells simultaneously expressing IL-4 and IL-17 (22). These Th17/Th2 hybrid cells were reported to be present in draining LNs and mLNs of mice with experimental autoimmune encephalomyelitis (EAE) that either had a CD4-specific Bcl11b-deficency or were treated with IL-4. In EAE, Th17 cells are pathogenic when they gain access to the central nervous system (CNS), contributing to CNS inflammation. EAE-induced Bcl11b-deficient mice showed a delayed onset and reduced disease severity, attributed to a redirection of T helper cells away from a draining LN/CNS route to the intestine. Even though an accumulation of Th17 cells in the intestine has been reported to cause colitis these mice were asymptomatic.

Th17-TO-Treg CELL PLASTICITY

A different approach was chosen to address Th17 cell plasticity by Carbo et al. (23). Computational modeling predicated a role of the nuclear receptor peroxisome proliferator-activated receptor gamma (PPARγ) in a phenotype switch from Th17-to-Treg cell in the murine gut. *In silico*, PPARγ activation of fully differentiated Th17 cells reduced IL-17 and RORγt, while initiating Foxp3 expression. *In vitro*, analysis confirmed these *in silico* predictions. Polarized Th17 cells treated with pioglitazone, a synthetic PPARγ agonist, induced Foxp3 and inhibited RORγt and IL-17A expression. This effect was not observed in PPARγ-deficient Th17 cells. Mice that received pioglitazone orally to activate PPARγ recovered from transferred colitis, with a phenotypic switch in transferred T cells, from a predominantly Th17 phenotype to an iTreg cell phenotype characterized by decreased IL-17 and RORγt and increased expression of Foxp3.

In humans, $IL\text{-}17^+$ $FOXP3^+$ T cells were identified in inflamed intestinal mucosa of patients with CD, but not in patients with ulcerative colitis or healthy controls (24). These Treg/Th17 cells shared characteristics of Th17 and Treg cells, expressing *RORC*

(encoding RORγt) and CD161 and showing Treg-typical suppressor activity *in vitro*. In addition, the majority these FOXP3$^+$IL-17$^+$ T cells expressed CCR6, a receptor that mediates homing to skin and mucosal tissues (56) and high levels of integrin α4β7 and CD103, markers for gut-homing potential. Analysis of the TCR repertoire suggested that FOXP3$^+$IL-17$^+$ cells develop from FOXP3$^+$ Tregs when exposed to inflammatory signals in the gut (24) and *in vitro* stimulation of FOXP3$^+$ circulating cells can result in IL-17 expression (57). In human tonsils obtained after routine tonsillectomy, up to 25% of the FOXP3$^+$CD4$^+$ T cell population can produce IL-17 upon activation (25). These FOXP3$^+$IL-17 producers are CCR6$^+$, co-express *RORC* and *FOXP3* and display cell-contact dependent suppressive properties (25). Whilst FOXP3$^+$IL-17$^+$ cells in the LP appear unique to areas of intestinal inflammation in CD (24), in tonsil they are much more readily identifiable (25). Together these observations suggest that heterogeneity in the GALT micro-environment can influence Th17–Treg plasticity. Intriguingly, the FOXP3$^+$IL-17$^+$ T cells from CD patients also express IFNγ, IL-22, and IL-21 (24), suggesting plasticity not just between Th17 and Treg, but with other CD4$^+$ effector states.

Th17/Tfh CELL PLASTICITY

In addition to a Th17-to-Th1 conversion, Th17 cells can acquire a Tfh cell phenotype (26), including expression of Bcl-6, the transcription factor that is both necessary and sufficient for the formation of Tfh (58–60). Tfh provide antigen-specific help to germinal center (GC) B cells via CD40L, IL-4, and IL-21 (61). The only GALT to contain GCs are the PP and mLNs, and within these structures Tfh act to select high-affinity GC B cells to exit the PP GC as long-lived IgA secreting plasma cells and memory B cells (62). An IL-17-fate reporter mouse was used to show that transfer of Th17 cells into a T cell deficient recipient could support high-affinity IgA production in the GCs of PPs by converting into Tfh cells (26). Interestingly, this conversion resulted in the loss of IL-17 expression and other Th17 associated features to enable these cells to effectively switch into Tfh cells in the PP. The physiological relevance of these ex-Th17^{Tfh} cells in host defense or in microbial homeostasis remains to be elucidated. But, the essential role for Th17 cells in the production of high-affinity IgA (26) suggests that this Th17 to Tfh cell switch is critical for IgA-dependent immune responses.

IgA production in the gut can occur outside the GC but the IgA species are low-affinity, with little somatic hypermutation (63). The production of GC-derived IgA is essential for the maintenance of bacterial communities in the gut. Mice that express a mutated form of activation-induced cytidine deaminase (AIDG23S) that allows class switch to IgA but not somatic hypermutation have alterations of the gut microbiota, suggesting that IgA derived from the PP GC response is essential for maintaining homeostasis of the gut microbiota (64). This demonstrates that high-affinity IgA promotes healthy microbiota, rather than target it for elimination like IgG derived from the LN and spleen (62). The production of immunoglobulin that nourishes commensal microbiota is one of the features that discriminates the function of GALT GCs from peripheral GCs, and may be one of the reasons that GALT GC T cells exhibit plasticity that is not observed in the GCs of lymph nodes and spleen. Selective IgA deficiency (OMIM 137100) is a common incidental finding amongst blood donors (with numerous reports since the 1970s), without any particular symptomatology originally appreciated [reviewed in Ref. (65)]. However, it has recently been reported that IgA-deficient persons have increased frequency of gastrointestinal and respiratory infections, and of allergy and autoimmunity (66).

Treg-TO-Tfh CELL PLASTICITY

In mice, Th17 cells are not unique in their ability to switch to a Tfh cell phenotype, also Foxp3$^+$ cells have been reported to be able to do this in the PP GCs (27, 28). The first study used adoptive transfer of CD4$^+$EGFP$^+$ cells from spleen or LNs of Foxp3–EGFP reporter mice into T cell deficient mice (27). Whilst 80% of Foxp3$^+$ T cells switched off Foxp3 expression in the PPs, about half of the transferred cells kept their Foxp3$^+$ profile in the LP of the small intestine as well as in the spleen. The ex-Treg^{Tfh} cells that form in the PP expressed Bcl-6 and also IL-21, CXCR5, CD40L, ICOS, PD-1, and CD28, all associated with Tfh cell function (61). Functionally, it has been shown that these ex-Treg^{Tfh} cells induce GC formation in PPs and facilitate IgA-producing cells in the gut (27). These findings appear in contrast with the work of Hirota et al. that identified ex-Th17^{Tfh} cells (26). In the study by Hirota et al., mucosal Foxp3$^+$ T cells transferred into TCRα$^{-/-}$ hosts did not differentiate into Tfh cells, induce GC B cells or IgA production. It remains unclear whether differences in the models (Tsuji: CD3ε$^{-/-}$ recipients; Hirota: TCRα$^{-/-}$ recipients) or source of cells transferred (Tsuji: spleen and LN, Hirota: mucosal origin) or intestinal environment of the transfer recipients explain the discrepancies. In a separate study, Takahashi et al. transferred iTreg cells into TCRα$^{-/-}$ mice and showed that these cells can lose Foxp3 expression and become Tfh in the PP (67). Intriguingly, transferred iTreg cells were more likely to lose Foxp3 expression in the PP than in the spleen, suggesting that the micro-environment of the gut affects the plasticity of Tregs. The conversion of Treg-to-Tfh in the gut is controlled, in part, by expression of microRNA-10a that suppresses Bcl-6 and Ncor2, thereby repressing of Treg-to-Tfh conversion in the gut (67).

Not all Tregs within the PP convert into Tfh, however, there is a regulatory population of CD4$^+$ T cells in GCs; T follicular regulatory cells (Tfr) (68–70). Tfr are the progeny of Foxp3$^+$CD4$^+$ cells that share phenotypic characteristics of Tfh cells, including CXCR5, PD-1, and Bcl-6 expression. In peripheral secondary lymphoid organs, Tfr control the size and output of the GC response (68–70). In PP GCs, Tfr control the quality of IgA produced, which in turn promotes diversity within the microbial community of the gut (28). Taken together, conversion of Treg-to-Tfh in the gut is finely balanced to ensure the ratio of Tfh to Tfr within the PP supports high-affinity IgA production and homeostasis of commensal bacteria.

Th2-TO-Tfh CELL PLASTICITY

The gastrointestinal helminth parasite *H. polygyrus* has been shown to give raise to Th1/Th2 hybrid cells but also been suggested give rise to Th2/Tfh hybrid cells (71). These hybrids were described as IL-4 producing CD4$^+$ cells expressing CXCR5, ICOS and PD-1, Bcl-6 and IL-21 and that localized in B cell follicles of mLNs

after *H. polygyrus* infection. Zaretsky et al. found similar IL-4$^+$CXCR5$^+$PD-1$^+$ Th cells after immunization with schistosomiasis soluble egg antigen (72). A later study using dual reporters for IL-4 and IL-13 and *N. brasiliensis* infection demonstrated that Tfh phenotype cells express IL-4 but not IL-13, whereas Th2 cells express both prototypic Th2 cytokines. Importantly, IL-4$^+$ Tfh cells are Bcl-6$^+$ and do not express high levels of GATA-3, suggesting that these cells are Tfh cells that produce IL-4 rather than *bona fide* Th2/Tfh hybrids (73).

CD4$^+$ T CELL INTO CYTOTOXIC CD4$^+$ T CELL CONVERSION

Among the CD4$^+$ IELs a population of cells has been described that lost its CD4-lineage transcription factor ThPOK and acquired the CD8-lineage transcription factor Runx3 (29, 30). The induction of Runx3 expression by CD4$^+$ T cells was associated with expression of CD8αα, the natural killer cell – and CTL-related molecule 2B4 (CD244) and Tbet (29). In addition, these cells expressed granzyme and the activation-induced degranulation marker CD107a (LAMP-1) (30). As well as closely resembling mature CD8$^+$ CTLs, these cells showed high cytolytic activity. At the same time, these cells lose their ability to produce IL-17 and to express RORγt (29, 30). Fate-mapping and adoptive transfer experiments by Mucida et al. showed that CD4$^+$ CTLs originate from ThPOK$^+$ naive CD4$^+$ T cells (30).

The loss of ThPOK together with the inability to produce IL-17 in the intestine had physiological implications on the development of colitis. Conditional deletion of ThPOK in CD4$^+$ T cells prior to their transfer into lymphopenic Rag$^{-/-}$ deficient mice enhanced the differentiation of CD4$^+$CD8α$^+$ CTLs in the gut of the recipients, which, as a consequence, saw a reduction in intestinal inflammation compared with controls (29). By contrast, the transfer of Runx3-deficient CD4$^+$ T cells resulted in an exacerbated intestinal inflammation, but provided recipient mice with increased protection against infection with the enteropathogenic bacterium *Citrobacter rodentium* (29). A role of CD4$^+$ CTL cells in celiac disease is suggested by their restriction to MHC class II, their induction by dietary antigen, responsiveness to IL-15, and cytotoxic potential (30).

MECHANISMS OF CD4$^+$ T CELL PLASTICITY

Very little is known about the underlying mechanisms determining CD4$^+$ T cell plasticity. It is likely that the same factors that are involved in T cell differentiation are implicated in a phenotype switch, but this remains largely untested.

The development of some CD4$^+$ T hybrid cells or ex-Th lineage cells seem to predominantly take place in the intestine, indicating that the micro-environment has a major influence on CD4$^+$ T cell plasticity and identity. For example, the micro-environmental cues contributing to a phenotype switch between Th17 and Tfh seem to be unique to the PP, as the plasticity of Th17 cells toward a Tfh cell fate was restricted to the micro-environment of PPs and did not occur in peripheral LNs (26). Likewise, transferred Foxp3$^+$ T cells lost Foxp3 expression and acquired Tfh features preferentially in the PPs (27, 65). Further evidence of the unique nature of the PP comes from *in vitro* stimulation assays in which APCs from PP and spleen generate different CD4$^+$ effectors (74, 75).

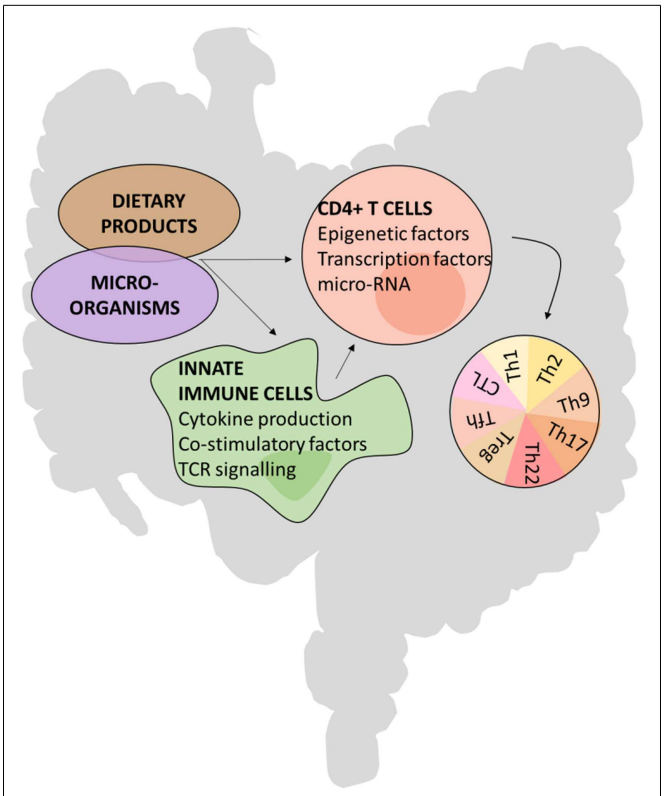

FIGURE 2 | Possible mechanisms of CD4$^+$ T cell plasticity in the gut.

Factors enabling CD4$^+$ T cell plasticity could, in part, originate from the microbiota or dietary products that effect CD4$^+$ T cells either directly or indirectly via innate immune cells. These extrinsic cues can alter transcription factor and microRNA expression and epigenetic markers such as histone modifications (**Figure 2**). For example, microRNA-10a dampens the conversion of Treg cells to both Th17 and Tfh cell fates (67). In addition, the cellular composition of the GALT, enriched in non-conventional lymphocytes such as ILCs, IEL populations of TCRγδ$^+$, and CD8αα$^+$TCRαβ$^+$ T cells and mucosal associated innate NKT cells, is substantially different compared with other secondary lymphoid organs and will contribute to a different environment in which T cells encounter antigen and are maintained.

MICROBIOTA

The nature of antigen or metabolic products provided by the gut microbiota plays a role in T cell plasticity. There are a number of examples where CD4$^+$ T cell subsets are expanded in response to particular microbial species and their products. Using germ-free mice, a mixture of 17 *Clostridia* species were selected from human gut flora for their ability to induce mucosal Tregs (76). Introduction of these 17 strains mitigated colitis and allergic diarrhea models (76). This protective effect is mediated by short chain fatty acids, products of these 17 bacteria (and of *Bacteroides fragilis*, also shown to induce Tregs) (77). The microbiome is shaped, in part by induced Tregs, and mice lacking induced

Tregs are more susceptible to colitis and asthma (78). Thus the gut microbiome is implicated in the protection of autoimmunity and autoinflammation both in the gut and at distant sites. Recent work provides evidence for a symbiosis between $CD4^+$ T cell subsets and the microbiome (28). Lack of Tfr resulted in poor quality IgA production and a limited biodiversity of the microbiome. The presence of Tfr permitted a more diverse microbiome including a larger representation of non-pathogenic *Clostridia* (28), which establishes a positive feedback loop between $CD4^+$ cells and the microbiome mediated through SCFA, Foxp3 expression, and IgA production. This narrowing of the microbiome and subsequently SCFA deplete environment might explain the association between IgA deficiency and allergy and autoimmunity (66). Taken together, these studies provide evidence that the gut microbiota and $CD4^+$ T cell fate within the gut are inter-dependent, with each affecting the composition of the other.

$CD4^+CD8\alpha^+$ CTLs are absent from the intestines of germ-free mice and of mice mono-colonized with segmented filamentous bacteria (SFB), but appear in the intestines following reconstitution with specific non-pathogenic microorganisms (30). Little is known about the mechanisms how antigens originating from microbiota induce a phenotype switch. However, data on Th17 cell differentiation indicate that SFB colonization promotes Th17 cell commitment by the expression of inflammation-associated genes, such as the gene for serum amyloid A (SAA) (79). Some Th17 cells from murine gut have TCR specific for SFB-derived antigen (80). In addition, it has been shown that ATP derived from commensal bacteria activates $CD70^{high}$ $CD11c^{low}$ cells in the LP, leading to enhanced differentiation of Th17 cells (81). Therefore, changes in the concentration of SAA or ATP could either induce a switch toward Th17 phenotype or destabilize Th17 cell commitment toward another phenotype.

DIETARY PRODUCTS

Dietary products, mainly after being processed by microorganisms, can induce phenotype switch. PPARγ has been identified to induce a Th17-to-Treg cell switch (23). This nuclear receptor regulates fatty acid storage and glucose metabolism. Therefore, consumption of food containing certain fatty acids may promote a phenotype switch from Th17-to-Treg cells. Several dietary products have been reported to promote or inhibit lineage commitment [reviewed in Ref. (2)]. It is likely that these factors are also candidates to induce a phenotype switch of $CD4^+$ T cells. In addition, dietary products could influence a phenotype switch by affecting metabolic and signaling pathways and epigenetic status.

INNATE IMMUNE CELLS: PROVIDERS OF COSTIMULATION AND CYTOKINES

Innate immune cells are abundant in the GALT. They have the potential to influence $CD4^+$ T cell plasticity via their determination of the micro-environment via the secretion of soluble mediators, expression of co-stimulatory molecules, and via their potential to act as APCs. Mucida et al. demonstrated that continuous activation of $CD4^+$ T cells by oral administration of an antigen is necessary for a phenotype switch to $CD4^+$ CTLs (30). It has been reported previously that antigen dose and peptide/TCR affinity can influence Th commitment (82).

Co-stimulatory factors have been shown to play a role in Treg-to-Tfh plasticity. Blocking the interaction of CD40, expressed on APCs such as B cells and DCs, with CD40L, found on T cells, is able to prevent a phenotype switch from Treg cell to Tfh cell (27).

The role of cytokines in Th17 cell plasticity has been studied by several investigators but has not yet been fully elucidated. However, it is clear that IL-23-dependent pathway does play an important role. In *Helicobacter hepaticus*-induced typhlocolitis, mRNA levels for IL-23p19 were elevated after bacterial inoculation and *ex vivo* $IL\text{-}17A^+$ cells isolated from the colitic intestine expressed both subunits of the IL-23R, indicating that IL-23 acts on Th17 cells to induce a program resulting in IFNγ production (18). T cell transfer studies also showed that IL-23 is required for the appearance of $IL\text{-}17A^+IFN\gamma^+$ double-producing T cells in the intestine. Transferred naive $CD4^+$ T cells developed to Th1/Th17 hybrid cells in $Rag^{-/-}$ mice, but not in $p19^{-/-}$ $RAG^{-/-}$ mice [lacking IL-23; (21)]. These studies were extended by Ahern et al. demonstrating that in the intestine the emergence of $IL\text{-}17A^+IFN\gamma^+$ double-producing Th cells, but not IL-17A single producing Th cells, requires T cell-intrinsic IL-23 signaling by transferring $IL\text{-}23R^{-/-}CD45RB^{high}$ $CD4^+$ T cells into full hosts (17). In humans, $CD161^+CD4^+$ T cells from CD patients readily produce IL-17 and IFNγ upon stimulation with IL-23, whereas, in healthy subjects, IL-1β was required alongside IL-23 (16).

The role of IL-12, the primary cytokine inducing Th1 cell differentiation, in mediating Th17-to-Th1 cell conversion remains controversial. *In vitro*, obtained results differ from those data being observed in *in vivo* models. Upon transfer of naive $CD4^+$ T cells, Th17/Th1 cells were detected in the mLNs of IL12p35-Rag-double deficient mice (lacking IL-12), demonstrating that IL-12 is negligible in Th1/Th17 induction in the intestine (21). In contrast, Lee et al. showed *in vitro* that in both IL-23 and IL-12 are able to switch off IL-17 and enhance IFNγ production in a STAT4- and Tbet-dependent manner. It has also been shown that *ex vivo* Th17 cells can be converted into a Th1-like phenotype following IFNγ-induced expression of Tbet and acquisition of IL-12Rβ2 surface expression (83, 84). Lee et al. investigated the effect of additional cytokines on Th17 cell plasticity and found that Th17 cells require TGFβ for sustained expression of IL-17F and IL-17A (20).

Although the precise role of IL-23 in the Th17-to-Th1 cell conversion requires further investigation, it does not seem to play an essential role in a phenotype switch from Th17 to Tfh cell. Th17 cells can give rise to Tfh cells in IL-23-competent and -deficient mice (26). For a phenotype switch from Tregs to Tfh cells it has been shown that the cytokine IL-6 can to down-regulate Foxp3, and this may be relevant to Treg plasticity in the IL-6 rich PPs (85).

TRANSCRIPTION FACTORS

Transcription factors maintain and instruct lineage programs by complex mechanisms, often involving several cooperating transcription factors, and their binding and access to specific DNA sequences. The expression of lineage determining transcription factors is induced by multiple factors in the micro-environment, including cytokines. Their regulation falls outside the scope of this review, but their interactions are of importance in maintaining Th

identity and are likely to play a prominent role in ultimately generating hybrid Th cells and Th identity conversions. Co-expression of IFNγ and Th2-associated cytokines in the same T cell seems to be enabled by signals that keep both Tbet and GATA-3 expression in balance, which is in contrast to studies examining chromatin modification at *Tbx21* and *Gata3* (86, 87).

The loss of a single transcription factor can tip the balance in favor of an alternative lineage. In Bcl11b-deficient mice, GATA-3 expression in Th17 cells is unrestrained, resulting in GATA-3-mediated IL-4 production (22). This change in cell phenotype feeds back to the micro-environment, and has further implications for T cell biology. For example, the cytokine mix produced by Th2/Th17 hybrid cells triggers gut-imprinting properties in DCs. IL-4 together with GM-CSF enhance the expression of the enzyme RALDH2 in DCs (88, 89), leading to elevated levels of RA (90) that imprint gut-homing properties on T cells, such as α4β7 and CCR9 (91). Similarly, the termination of ThPOK in mature $CD4^+$ T cells enables the de-repression of the CTL program (30), resulting in MHC class II restricted $CD4^+$ T cells with a cytotoxic effector function. This process can be further promoted by Runx3 (29).

The factors responsible for carefully balancing two transcriptional programs within a single T cell may be present together from the moment of T cell differentiation or may be induced later. *In vitro*, data have shown that expression of one differentiation program is often found to be mutually exclusive with factors from another (55). Yet, differentiation of some programs are characterized by co-expression of two pathways from the start, such as seen with iTreg differentiation in which T cells co-express Foxp3 and RORγt from the start (25). These *in vitro* observations are often not recapitulated *in vivo* and vice versa. This highlights missing links in our understanding of which factors are required to determine full Th effector cell differentiation, which factors enable T cells to postpone the identity decision, as well as those that can still interfere at later stages.

EPIGENETICS

The observed stability or plasticity of Th subsets is governed by the epigenetic regulation of the key transcription factors and cytokines and their access to downstream genes (92). Whether a particular gene is poised for expression is controlled by the chromatin structure and histone or DNA modification state. Wei et al. evaluated the histone H3 methylation status over the entire genome in a variety of defined Th subsets (87). Promoter regions for *Tbx21* (encoding Tbet) in Th1 cells and *Rorc* (encoding RORγt) in Th17 cells displayed a permissive methylation state (H3K4me3) associated with full expression of these master regulators in each lineage. However, the promoter region for *Tbx21* in Th17 cells had a bivalent status characterized by H3K4me3/H3K27me3, reflecting the relative instability of these cells and their potential to acquire another Th cell phenotype. In addition, it has been shown that treatment of naïve T cells under Treg polarizing conditions with the fatty acid, butyrate, enhances permissive acetylation in the promoter region of the *Foxp3* locus. Butyrate, being a large bowel fermentation product, provides a link between microbial products and Treg cells by epigenetic mechanisms. Beyond histone modifications, miRNAs offer a further tuning of gene expression and $CD4^+$ phenotype, as illustrated earlier.

FUNCTION

$CD4^+$ T cell plasticity is important in the resolution of infections but can contribute to immunopathogenesis. The ability to switch or combine effector phenotypes can generally be seen as a very useful feature in the arsenal of the adaptive immune system. It is an additional tool providing flexibility to adequately control rapidly changing microorganisms. It allows the retention of TCRs with a useful track record while displaying a high degree of flexibility and adaptation depending on local environmental cues. Therefore, a phenotype switch may facilitate the ability to respond more quickly to changing immunological challenges and to successfully terminate immune responses after pathogen clearance. This flexibility may be especially important and useful at epithelial interfaces, where luminal content is constantly changing and the largest population of microorganism is encountered.

The ability to switch from one phenotype to another or obtain features from two effector programs increases the efficiency of a response. Combining effector mechanisms would enable the control of complex organisms that have evolved ways to deal with one predominant effector subset. This implies that effector programs can co-exists within one T cell, while the co-existences of different effector subset within the same micro-environment may be harder to achieve and maintain. It is worth noting that reports on Th cells displaying a hybrid phenotype highlight that such cells are less effective in dealing with situations where one part of their identity is required compared with the single identity T cells. This indicates that each identity is weakened by the presence of the transcriptional program of the other, a feature that could be abused by microorganisms. Conversely, producing these "weakened" T cells may be method of peripheral tolerance – in an inflamed milieu, T cells are stimulated under polarizing conditions and develop a single identity, whereas non-inflamed milieu offers conditions less favorable to any single identity and the "weakened" T cells are tolerized.

An additional physiological role for $CD4^+$ T cell plasticity is the gaining of specific functional features that require aspects of the previous full identity combined with a very specific trade. This is the case for Tfh cells that can develop from committed Th17 or Treg cells. In case of ex-$Th17^{Tfh}$ cells, they represent a subset of Tfh cells that are specialized to promote high-affinity IgA production in the GCs of PPs. A similar specialized role in GC B cell support is likely the case for ex-$Treg^{Tfh}$. These Tfh adopters have only been found in the PP and only support IgA production, demonstrating that this plasticity is unique to the gut environment.

Lastly, the transcriptional programs of each T cell effector subset may not be compatible with long-term survival. Effector cells that switch to a Th1 cell phenotype, may be maintained better as memory cells due to the activity mediated by Tbet. Recent reports describe a role of Tbet expression gradient as a regulator of $CD4^+$ T cell memory formation, with highly Th1-polarized $Tbet^+$ cells displaying end-effector features and a short lifespan, whereas Th cells with a lower expression of Tbet are able to enter the long-lived memory T cell pool (93, 94). This is to some degree mimicked by the finding that long-lived ex-Th17 cells acquired Tbet expression, albeit at levels lower than their Th1 counterparts.

From a therapeutic viewpoint, contemplating a deliberately induced phenotype switch could be used to up- or down-regulate

the different effector arms of the adaptive immune response. Dampening $CD4^+$ T cell function would be useful for autoimmune disorders including CD and multiple sclerosis. In a colitis model, oral pioglitazone induces a switch from Th17 to an iTreg phenotype, improving disease and in EAE (a model of MS) pathogenic Th17 cells can be redirected to gut, again improving disease. Enhanced $CD4^+$ T cell responses could improve cancer surveillance and clearance. Whilst phenotypic switching appears to occur in very specific circumstances, some of these conditions are encountered spontaneously in healthy animals. Thus strategies to induce phenotype switching, with minimal side effects or co-morbidities, might be identifiable.

CONCLUSION

The nuanced view of Th differentiation suggested that within any $CD4^+$ effector population there are varying ratios of lineage determining transcription factors in each cell, with correspondingly graded cellular phenotypes. In GALT these ratios integrate a number of different signals – microbial products, dietary influences, costimulation, cytokines, and epigenetic modifications. Variation in these signals and their interpretation is the substrate for heterogeneity of $CD4^+$ T cells. As these microbial and dietary cues are unique to GALT, perhaps even unique to the most luminal GALT, such as PP, they could together provide a micro-environment permissive for plasticity, in contrast to other secondary lymphoid organs, where plasticity is less frequently reported and where much of the rigid *in vivo* definitions of the $CD4^+$ subsets were derived. Whether the phenotypic switches described herein are unique to an *in entero* environment is unknown. It is possible that similar blurring of $CD4^+$ subsets occurs at other mucosal sites, balancing tolerance, protection, and immunopathology with the need to maintain a diverse microbiome. To understand the precise factors underlying plasticity is relevant for our understanding of host (and microbiomial) health, host defense, pathogenesis of various gastrointestinal diseases and also for development and optimization of vaccines, where an enforced Tfh phenotype is desired.

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Memory T follicular helper CD4 T cells

J. Scott Hale* and Rafi Ahmed

Department of Microbiology and Immunology, Emory Vaccine Center, Emory University School of Medicine, Atlanta, GA, USA

Edited by:
Dragana Jankovic, National Institutes of Health, USA

Reviewed by:
António Gil Castro, University of Minho, Portugal
Remy Bosselut, National Cancer Institute, USA

****Correspondence:***
J. Scott Hale, Department of Microbiology and Immunology, Emory Vaccine Center, Emory University School of Medicine, 1462 Clifton Road, Atlanta, GA 30322, USA
e-mail: jshale@emory.edu

T follicular helper (Tfh) cells are the subset of CD4 T helper cells that are required for generation and maintenance of germinal center reactions and the generation of long-lived humoral immunity. This specialized T helper subset provides help to cognate B cells via their expression of CD40 ligand, IL-21, IL-4, and other molecules. Tfh cells are characterized by their expression of the chemokine receptor CXCR5, expression of the transcriptional repressor Bcl6, and their capacity to migrate to the follicle and promote germinal center B cell responses. Until recently, it remained unclear whether Tfh cells differentiated into memory cells and whether they maintain Tfh commitment at the memory phase. This review will highlight several recent studies that support the idea of Tfh-committed CD4 T cells at the memory stage of the immune response. The implication of these findings is that memory Tfh cells retain their capacity to recall their Tfh-specific effector functions upon reactivation to provide help for B cell responses and play an important role in prime and boost vaccination or during recall responses to infection. The markers that are useful for distinguishing Tfh effector and memory cells, as well as the limitations of using these markers will be discussed. Tfh effector and memory generation, lineage maintenance, and plasticity relative to other T helper lineages (Th1, Th2, Th17, etc.) will also be discussed. Ongoing discoveries regarding the maintenance and lineage stability versus plasticity of memory Tfh cells will improve strategies that utilize CD4 T cell memory to modulate antibody responses during prime and boost vaccination.

Keywords: T follicular helper cells, memory T cells, Bcl6, CXCR5, helper T cells

INTRODUCTION

Effective B cell responses to infectious diseases or immunization require the assistance of CD4+ helper T cells. A specialized subset of CD4 T cells named T follicular helper (Tfh) cells are required for providing this help to antigen-specific B cells. Without cognate Tfh help, activated B cells are unable to generate and maintain the germinal center response that is required for efficient somatic hypermutation of immunoglobulin genes and the selective processes that facilitate affinity maturation of antibodies (1, 2). Furthermore, the germinal center reaction is the origin of long-lived memory B cells and long-lived plasma cells that populate the periphery and bone marrow (respectively), and provide long-term antibody-mediated protection against (re)exposure to pathogens (3). Thus, Tfh cells play a critical role in the generation of effective and long-lived humoral immune responses to antigens (1).

T follicular helper cells were first identified as a subset of CD4 T cells isolated from human tonsils (4, 5). These cells expressed the B cell follicle homing chemokine receptor CXCR5 and the inducible costimulator (ICOS), and localized within the germinal center (4, 5). Furthermore, these human tonsil CXCR5+ cells (compared to CXCR5−CD45RO+ T cells) efficiently promoted production of class switched immunoglobulin (Ig)G and IgA in T cell:B cell co-culture assays (4–6). Interestingly, CXCR5+ CD4 T cells from human blood, which were presumed to be the memory counterparts of CXCR5+ cells in tonsils and lymph nodes, did not efficiently produce the Th1 signature cytokine IFNγ or the Th2 cytokines IL-4, IL-5, and IL-13 (5). Together, these studies suggested that the CXCR5+ Tfh cells represented a novel subset of helper T cells with the specific functions of providing help for B cell responses and that are distinct from Th1 and Th2 cells. Since these initial seminal reports describing Tfh cells, extensive studies have demonstrated that while Tfh cells share certain similarities with Th1, Th2, and Th17 cells (depending upon context of infection or vaccination), these cells have unique developmental requirements and distinct phenotypic, homing, and functional qualities compared to other T helper cell lineages (Th1, Th2, Th17, Treg) (1).

Upon activation with cognate antigen by dendritic cells, antigen-specific CD4 T cells can differentiate to become various types of effector CD4 T cells with specific roles in promoting anti-pathogen immune responses (**Figure 1**). Early differentiation toward the Tfh lineage requires ICOS expression and signaling to induce expression of the transcriptional repressor Bcl-6 (7). Bcl6 is required for Tfh cell generation, maintenance, and function, establishing Bcl6 as a central regulator in Tfh cell lineage development (**Figure 1**) (8–10). Bcl6 expression promotes Tfh differentiation, at least in part by suppressing transcription of the transcriptional regulators Tbet, RORγt, GATA3, and Blimp-1 (8–10), and through other potential mechanisms, such as the repression of microRNAs (10). In addition, cytokines such as IL-6 and IL-21 (11), and other molecules such as SAP are important for Tfh differentiation and function (12, 13). Importantly, interactions with cognate B cells were required for amplifying the expression of Bcl6 for the maintenance of the Tfh phenotype during the immune response (7). During Tfh differentiation, Bcl6 plays an important role in suppressing Blimp1 (8), which is a regulator of Th1, Th17, and Th2 lineage cells (1). Thus, the promotion of Bcl6 coupled to

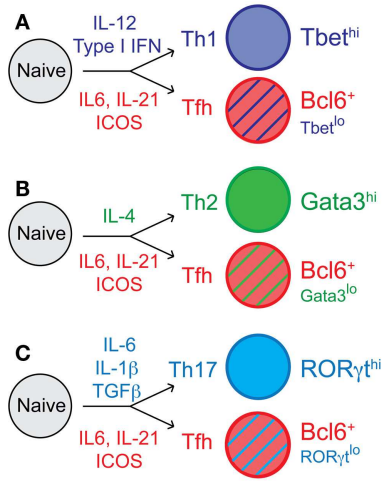

FIGURE 1 | T follicular helper cell differentiation and context-dependent Tfh cell heterogeneity. Following activation of naïve CD4 T cells, cells proliferate and undergo fate decisions in response to cytokines and other differentiating factors. Tfh cell differentiation is influenced by IL-6 and IL-21, and dependent upon ICOS signaling for expression of the transcription factor Bcl6. Cytokines including IL-12, IL-4, IL-1β, and many others, direct **(A)** Th1, **(B)** Th2, and **(C)** Th17 cell differentiation, respectively. The context-dependent cytokine milieu also influences Tfh cell differentiation; thus, Tfh effector cells can express some low/intermediate levels of the transcription factors Tbet, Gata3, and RORγt, which are associated with the **(A)** Th1, **(B)** Th2, and **(C)** Th17 cell lineages, respectively.

the repression of Blimp1 plays a role in the differentiation, function, and possibly the stability of Tfh cells in relation to other T helper cell subsets. Additional transcription factors such as Maf (14, 15), Ascl2 (16), and others play important roles in Tfh cell differentiation and/or function (1).

T follicular helper cells have developmental requirements that differ from those that promote Th1, Th2, and Th17 effector cell development (1). However, depending upon the type of infection (viral, helminth, fungal, etc.) or immunization and the inflammatory environment that is generated, Tfh cells can express low to intermediate levels of Tbet, Gata3, or RORγt (**Figure 1**) (17–20). This intriguing context-dependent transcription factor expression in Tfh cells results in a variety of Tfh cell subsets that can express low levels of specific cytokines that can influence antibody class switching (17, 21, 22). Thus, studying the complex relationship between Tfh cells and their non-Tfh cell counterparts, and whether they remain phenotypically and functionally distinct throughout the immune response and beyond is critical to understanding Tfh cell commitment and flexibility and to determining their biologically important roles during specific types of immune responses.

LINEAGE-COMMITTED MEMORY T HELPER CELLS

The adaptive immune system responds to infectious challenge with two major goals. The first goal is to generate sufficient numbers of antigen-specific effector cells to limit and clear the pathogen. The second priority is to provide long-lasting immunity that will defend the host from subsequent exposure to the pathogen (23). The activation-driven proliferation and lineage differentiation of CD4 T cells *in vivo* is accompanied by the progression of memory differentiation. Following clearance of antigen, the majority (approximately 90–95%) of antigen-specific effector T cells undergo apoptosis, leaving behind a population of memory cells. In some experimental models, antigen-specific CD4 memory T cells gradually decline over long periods of time (24, 25). For example, *Listeria monocytogenes* infection-induced memory CD4 T cells are present at relatively high frequencies 90 days post-infection; however, by approximately 250 days post-infection, the population has largely disappeared from the spleen and lymph nodes (25). In contrast, human studies reveal that long-lived vaccinia-specific memory CD4 T cells are relatively stable for at least several decades after smallpox vaccination (26, 27).

Memory T cells possess many important features compared to their naïve CD4 T cell precursors. First, antigen-specific memory cells are found in increased numbers relative to their naïve antigen-specific precursors, providing better coverage and a more rapid cellular response upon pathogen rechallenge. Second, memory cells are not restricted to blood circulation and secondary lymphoid organs, but instead may also traffic to and reside in non-lymphoid tissues, where they may rapidly exert effector functions if their specified pathogen gains entry to that particular anatomical site. Third, memory T cells have undergone changes in cell-intrinsic programing, allowing them to rapidly recall their effector functions, such as prompt expression of specific effector cytokines, chemokines, and cytotoxic molecules. Finally, memory cells are long-lived, and a central feature of their longevity is dependent on their ability to undergo homeostatic proliferation in the absence of antigen (23, 28).

Combining the study of T helper lineage differentiation and T cell memory differentiation *in vivo* following vaccination or infection is incredibly complex. However, it provides the opportunity to gain vital understanding into the heterogeneity and lineage commitment and flexibility of the resulting antigen-specific memory CD4 T cells that will be informative for ongoing and future vaccine discovery/development efforts. It has become clear that among the vast heterogeneity of memory CD4 T cells, many memory cells demonstrate commitment to a previously defined T helper lineage. The existence of Th1-commited long-lived memory CD4 T cells was demonstrated in BAC transgenic mice that used a reporter to indicate transcription of the *Ifng* gene. In this study, Harrington et al. demonstrated that these memory cells were derived from the effector Th1 cells, and rapidly recalled IFNγ expression at the effector phase (29). Several other studies similarly found that subsets of LCMV-specific and *Listeria*-specific memory CD4 T cells with distinct phenotypes were committed Th1 memory cells that recalled IFNγ expression and expression of other Th1 effector molecules (20, 30, 31). One study utilized an IL4 IRES EGFP reporter mouse to demonstrate that Th2 effector cells (EGFP+) generated from *N. brasiliensis* infection could provide anti-parasite protective immunity after adoptive transfer into immunocompromised recipient mice and 30 days resting before parasite challenge (32). Similarly, *Trichuris*-specific Th2 memory cells recall their Th2 effector functions and mediate anti-parasite immunity (33). While Th17 CD4 T cells generated by intranasal *Listeria* infection (a Th1 pathogen) do not form memory cells (25), *Candida* and other fungal vaccines,

as well as other conditions have been shown to induce Th17 memory cells *in vivo* (34–36). Together, these studies demonstrate the characteristics and programs of polarized effector Th1, Th2, and Th17 cells that are generated early during effector differentiation are preserved in resting memory cells. Importantly, these effector programs are recalled after reactivation *in vivo* to infectious challenge in an antigen-specific manner, and with the appropriate T helper effector response to effectively eliminate the pathogen.

T FOLLICULAR HELPER MEMORY CELLS

The establishment of Tfh cells as an independent effector T helper subset, and the factors that drive Tfh differentiation being defined, provides a strong rationale for exploring whether Tfh cells that progress to become memory cells maintain their Tfh attributes following resolution to the immune response. However, given the potential flexibility/plasticity of Tfh cells toward repolarization (37), one might predict that Tfh cells generate non-committed memory CD4 T cells. Several fundamental questions exist regarding the relationship of Tfh cells and memory cells. First, do Tfh cells survive to become memory cells? Second, do memory cells derived from Tfh cells maintain their commitment/programing to recall Tfh effector cells, or, instead possess pluripotency/plasticity to become cells of other T helper lineages? The answer to these questions provides profound insight into the importance of how Tfh differentiation during primary immune responses to natural infection and vaccination have the potential to influence secondary antibody responses. Several recent studies have provided insight into the existence and characteristics of memory Tfh cells and their capacity to recall Tfh-specific effector functions following reactivation with antigen.

One early study investigating memory Tfh cells in mice described a population of CXCR5+ICOS+ cells in the draining lymph nodes 30–56 days following immunization with pigeon cytochrome C in adjuvant. Compared to day 7 Tfh effector cells, day 30 PCC-specific cells had decreased ICOS and OX40 expression. The authors reported that these were a subset of memory Tfh cells with enhanced recall capacity upon immunization 6–8 weeks after priming (38). However, this study further reported the persistence of antigen for more than 75 days after immunization and the maintenance of CD69 on these PCC-specific CD4 Tfh cells indicate that this experimental system promotes long-lived antigen depots. Thus, the CXCR5+ICOS^{lo} cells identified in this study cannot be clearly distinguished as true memory cells that survive in the absence of antigen (38).

A study by MacLeod and Marrack provided a stronger basis for the existence of memory CD4 T cells with accelerated Tfh function during recall responses (39). Their study demonstrated that on a per cell basis, antigen-specific memory CD4 T cells (compared to Ag-specific naïve cells) provided accelerated B cell responses and antibody class switching. Interestingly, this accelerated B cell helper capacity was contained within the CXCR5+ subset of memory CD4 T cells, resulting in higher OVA-specific IgG1 titers following adoptive cell transfer and followed by immunization. The authors suggested that CXCR5 chemokine receptor expression promotes their more rapid migration of reactivated memory CD4 T cells to B cell follicles, allowing them to provide accelerated help to the B cell response (39).

It was unknown whether Tfh cells were able to survive and become memory cells. To address this question, Weber et al. used protein immunization in CFA to induce TCR transgenic CD4 effector T cells, sorted them into CXCR5− and CXCR5+ subsets, and then adoptively transferred these into naïve recipient mice (40). Fourteen days later, adoptively transferred CXCR5+ effector cells had become CXCR5 low/negative. After resting these cells in naïve recipient mice for several weeks, a large proportion of transferred Tfh cells quickly recalled a Tfh phenotype within 2.5 days following re-immunization, expressing higher levels of PD-1, Bcl6, CXCR5, and IL-21 compared to non-Tfh cells and primary effector cells (40). While the time periods that these cells were rested in the absence of antigen were relatively brief, this study was highly suggestive that effector Tfh cells could be maintained in the absence of antigen and preferentially recall a Tfh phenotype. A study by Choi and colleagues addressing the fate commitment of Tfh cells demonstrated that day 3 LCMV-specific Tfh effector cells, following adoptive transfer into day 3 LCMV-infected (infection-matched) recipient mice, were maintained up to 45 days later and retained CXCR5 surface expression (41). An additional study demonstrated that CXCR5+RFP+ (from Bcl6 RFP reporter mice) OTII cells induced by Ova/CFA immunization can persist following adoptive transfer for 20 days, and upon OVA+CFA immunization preferentially recall Tfh cells in the draining lymph node (42). Together, these studies clearly demonstrate that not all Tfh effector cells are terminally differentiated and fated to die. Instead, some Tfh effector cells progress to become memory cells that have some similar features compared to their Tfh effector predecessors.

Our study investigated the differentiation of Th1 and Tfh cells following acute LCMV infection using the adoptive transfer and analysis of LCMV-specific CD4 T cells (SMARTA TCR transgenic) induced by acute viral infection (20). Following the clearance of acute LCMV infection and the corresponding contraction of virus-specific CD4 T cells, both Th1 and Tfh memory subsets are maintained at relatively stable numbers for approximately 60–150 days post-infection (20). Resting PD-1− and ICOS−CXCR5+Ly6c^{lo} memory CD4 T cells shared phenotypic and gene expression similarities to CXCR5+Ly6c^{lo} effector Tfh cells, suggesting a direct lineage-relationship between these populations at the different stages of the immune response. Adoptive transfer of CXCR5+ memory subsets followed by LCMV Armstrong challenge resulted in Tfh secondary effector cells with ability to rapidly recall a Tfh effector phenotype and promote the generation of germinal center B cells. In addition, when CXCR5+ memory cells were transferred to B cell deficient recipient mice that were then infected, a large proportion of the resulting effector cells recalled and sustained a Tfh-like phenotype, while primary effector cells generated from naïve (uncommitted) SMARTA cells did not. These latter results indicate that CXCR5+ memory cells have acquired and maintained a Tfh-biased cell program relative to their naïve cell counterparts (20). Interestingly, while there was some apparent flexibility by some CXCR5+ memory cells to generate CXCR5− Th1-like secondary effector cells, cell-intrinsic restrictions impaired the Th1 effector program, resulting in poor granzyme B and IFNγ expression. The results from this study argue in favor of Tfh lineage commitment within the CXCR5+ Tfh memory cell population (20).

In contrast to these many studies that report the existence of memory Tfh cells that promptly and preferentially recall Tfh phenotype and function upon rechallenge, one study reported that *L. monocytogenes* specific (identified by MHC class II tetramer) CXCR5+ memory CD4 T cells are pluripotent, promoting the recall of both Tfh cells and non-Tfh (Th1) cells (31). Another study of influenza infection in IL-21 GFP reporter mice showed that the adoptive transfer of polyclonal CXCR5+GFP+ Tfh CD4 effector cells gave rise to memory cells with sufficient plasticity to generate secondary effectors of both Tfh cells and non-Tfh cells. Thus, Luthje et al. conclude that Tfh effector cells are "uncommitted" regarding their T helper lineage (43). Interestingly, these studies both used adoptive transfer of polyclonal CD4 T cells (31, 43), while studies demonstrating relative Tfh commitment of CXCR5+ Tfh memory cells utilized TCR transgenic cells specific for a single epitope (20, 40, 42). Because strength and/or longevity of TCR signaling influences Th1 versus Tfh differentiation (44, 45) as well as memory CD4 T cell differentiation (46), individual TCRs within the Tfh effector population may confer varying degrees of lineage commitment in CXCR5+ Tfh memory cells. Going forward, it will be interesting to determine whether different TCRs, infection and immunization systems, and other factors play a role in the degree of plasticity versus commitment in memory Tfh cells.

There are many well described Tfh cell markers; however, there are relatively few markers useful for clearly distinguishing resting memory Tfh cells. Antigen-specific memory Tfh cells express CXCR5, but lack Bcl6, ICOS, and expression of many other Tfh molecules (20, 41, 47) (**Figure 2**). In addition, memory Tfh cells do not express CD69 (20), an indicator of ongoing antigen stimulation. A recent study demonstrated that folate receptor 4 (FR4), a surface receptor that is upregulated on Tfh cells but not Th1 cells during acute viral infection, is maintained on the surface of CXCR5+ memory Tfh cells (48). To date, the best marker for identifying memory Tfh cells is CXCR5 (18, 20, 41, 47) (**Figure 2**); however, not all CXCR5+ memory cell subsets exhibit specific Tfh function upon reactivation (47). **Figure 2** shows some of the phenotypic markers associated with the memory Tfh cell phenotype.

It has become clear that Tfh effector cells can become memory Tfh cells with cell-intrinsic programs that promote the recall of Tfh cells upon reactivation. It is therefore possible to propose models of T helper cell differentiation wherein naïve CD4 T cells give rise to Tfh and other T effector lineage cells (Th1, Th2, or Th17 depending upon context of the pathogen or inflammation), and these effector cells give rise to memory cells that strictly maintain their lineage commitment and recall their lineage upon reinfection or boosting with antigen (**Figure 3A**). However, memory T cell populations are characterized by a vast degree of phenotypic and functional heterogeneity. A second and more comprehensive model would suggest that as effector cells differentiate, individual cells acquire varying degrees of programing toward the Tfh or other T effector lineage cells that can be stably maintained in resting memory cells (likely through epigenetic mechanisms) (**Figure 3B**). The resulting population of antigen-specific memory cells would then contain a wide spectrum of cells with varying degrees of lineage commitment versus pluripotency/plasticity (**Figure 3B**). This latter model may account for why some studies report lineage commitment within Tfh cell subsets, while others studies report relative non-commitment or plasticity. Future mechanistic studies will provide improved insight into the heterogeneity of memory CD4 T cells in relation to their T helper lineage commitment and recall potential. Furthermore, determining whether Tfh memory cells are homeostatically maintained over time at very late memory timepoints remains to be explored.

HUMAN T FOLLICULAR HELPER MEMORY CELLS

The original reports describing CXCR5+ Tfh cells in human tonsils had differing ideas regarding the existence of memory Tfh cells. One report described that tetanus-antigen-specific proliferation was induced in CD4+CD45RO+CXCR5− but not CD4+CD45RO+CXCR5+ cells, thus suggesting that Tfh cells likely disappear along with germinal centers, and are prone to apoptosis due to their high levels of Fas expression (4). In contrast, the other report showed that unlike the activated tonsillar CXCR5+ CD4 T cells that were CD69+HLA-DR+ICOS+, circulating CXCR5+ cells in human blood are CD69−HLA-DR−ICOS−, and hypothesized that these blood CXCR5+ cells represent memory Tfh cells (5). Since these initial observations, whether activated human Tfh cells become memory T cells, and whether

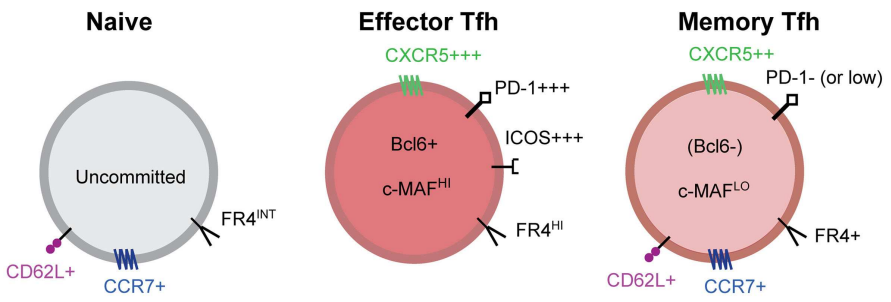

FIGURE 2 | Markers of effector and memory Tfh cells. Tfh effector CD4 T cells express high levels of the chemokine receptor CXCR5, the inhibitory receptor PD-1, ICOS, the transcriptional repressor Bcl6, the transcription factor c-Maf, and many other molecules. After antigen clearance, resting memory Tfh cells no longer express Bcl6, ICOS, and IL-21 and many other Tfh associated molecules. Tfh memory cells are characterized by intermediate expression of CXCR5 and other Tfh related molecules and the absence of activation dependent molecules. Bcl6 expression is not detected in memory Tfh cells; however, Maf and other transcription factors are maintained at low/intermediate levels. PD-1 is absent from antigen-specific memory Tfh cells in mice, however, human CXCR5+CXCR3− memory Tfh cells maintain low levels of PD-1 expression that is not dependent on TCR signaling. Folate receptor 4 (FR4), a molecule that is highly expressed on effector Tfh cells, is maintained on CXCR5+ memory Tfh cells.

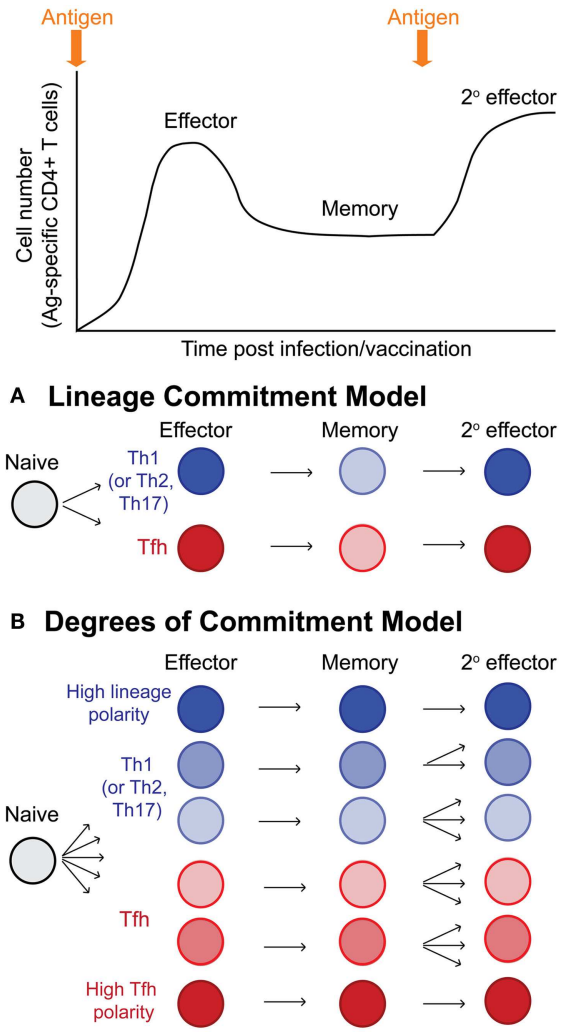

FIGURE 3 | Models of T helper lineage commitment in memory CD4 T cells. Following activation, naïve CD4 T cells undergo dramatic antigen dependent proliferation to generate effector CD4 T cells. Following antigen clearance, the majority of effector cells undergo apoptosis, leaving behind a pool of antigen-specific memory cells. Upon reinfection or antigen boost, memory cells become reactivated and proliferate, generating secondary effector cells. **(A)** Potential model for lineage commitment of Tfh memory cells. In this model, antigen-specific CD4 T cells differentiate to become either lineage-committed Tfh cells or lineage-committed effector cells of other lineages (either Th1, Th2, or Th17 depending upon the context of infection or inflammatory stimulus). Resting memory Tfh and non-Tfh cells would maintain their lineage-specific programing, and upon reactivation, would faithfully recall their previously defined T helper lineage phenotype and functions. **(B)** In this second model, antigen-specific CD4 T cells differentiate toward either Tfh or non-Tfh (Th1, Th2, or Th17) effector cells; however, depending upon the integration of many different stimuli (TCR signals, inflammation, interactions with various antigen-presenting cells, etc.), individual effector cells become transcriptional and epigenetically programed with varying degrees of polarity toward Tfh or non-Tfh cell lineages. Following antigen clearance and T cell contraction, the antigen-specific memory pool is highly heterogenous, being comprised of cells with varying degrees of lineage commitment and T helper lineage-specific recall potential, including highly committed Tfh memory cells, more plastic/pluripotent Tfh and non-Tfh (Th1, Th2, and Th17) memory cells, and highly committed non-Tfh (Th1, Th2, and Th17) memory cells.

they maintain their Tfh function upon reactivation has remained unclear. Recent reports have shed light on the nature of human CXCR5+ memory Tfh cell ontogeny and function (18, 37, 47).

One study described a population of circulating human CXCR5+ central memory CD4 T cells that expressed CXCL13 and promoted B cells to undergo plasma cell differentiation and Ig secretion (37). Another recent study investigating circulating CXCR5+ CD4 T cells in human blood revealed that these cells share functional characteristics with Tfh cells. CXCR5+ cells promoted the isotype switching and antibody production of IgG, IgA, and IgE isotype switched antibodies in T:B cell co-culture experiments, while CXCR5− cells did not promote switched antibody production (18). Their study further categorized the CXCR5+ population into CXCR3+CCR6− (Th1-like), CXCR3−CCR6+ (TH17-like), and CXCR3−CCR6− (Th2-like) subsets. While CXCR5+CXCR3+CCR6− cells did not provide help in these co-culture assays, CXCR5+CXCR3−CCR6+ cells promoted high levels of IgG and IgA antibodies, while CXCR5+CXCR3−CCR6− cells promoted high IgG and IgE, and intermediate levels of IgA. This study highlights the function of reactivated CXCR5+ CD4 T cells from peripheral blood to promote antibody production, and further defines the vast heterogeneity of CXCR5+ cells in relation to the Th1, Th2, and Th17 lineage of cells (18). The CD69− and ICOS− phenotype of these circulating CXCR5+ cells (prior to reactivation) suggests that they are resting memory cells (18). However, inability to identify antigen-specific cells and relate them to a known time of antigen exposure lead to difficulty in concluding whether they are indeed true memory Tfh cells that persist long-term in the absence of antigen and maintain their lineage characteristics in their resting state.

Locci et al. profoundly advanced the understanding of human Tfh memory cells and clarified their existence. Their study identified that blood CXCR5+CXCR3− cells that had a non-activated/resting phenotype could be stratified into either PD-1− or PD-1^{low} subsets (47). Interestingly, low PD-1 expression was stably maintained on this latter subset of cells during 20 day culture *in vitro* in the absence of TCR stimulation, suggesting that sorted CXCR5+PD-1^{low} cells express these molecules (PD-1 and CXCR5) as a part of the Tfh transcriptional program. Indeed, the transcriptional profile of these CXCR5+CXCR3−PD-1^{low} cells is similar (albeit reduced) to that of germinal center Tfh cells from human tonsils, and these cells promoted the highest IgG antibody production and plasmablast differentiation in T:B cell co-culture experiments compared to other subsets of CXCR5+ cells (47). Furthermore, characterization of tetanus-specific CD4 T cells in healthy human donors (identified using HLA tetramers bearing tetanus peptide), demonstrated the existence of this CXCR5+CXCR3−PD-1^{low} phenotype among resting antigen-specific memory cells (**Figure 2**). Together, these experiments define the phenotype and function of human memory Tfh cells and combine to fortify the idea that memory Tfh cells exist within the pool of resting human antigen-specific memory cells (47).

Defining strategies and markers to distinguish and characterize antigen-specific human Tfh effector and memory subsets is difficult, and is the focus of many recent and current lines of research (18, 47, 49–51). The vast heterogeneity of effector and memory CD4 T cells in human blood and other tissues has led

to difficulty in understanding what Tfh-like cell subsets serve as the best correlates of effective antibody responses, and what subsets actually provide the help for the B cell response *in vivo* (51). Some of the differences for Tfh and/or Tfh memory markers utilized in these various studies may result from the different types of infections or vaccinations studied, the timepoints analyzed (early after vaccination versus long-lived memory cells), and the varying analysis of "bulk" memory populations of unknown antigen-specificities versus analysis of tetramer+ pathogen specific memory cells (18, 47, 49–51). As better markers, reagents, and methods for studying antigen-specific Tfh responses become available, the developmental requirements and heterogeneity of human memory Tfh cells and their relationship to other T helper cell subsets will be better understood. This will open doors to more clearly define the specific roles that Tfh memory cells play during recall responses by human Tfh cells during boosting or in response to natural infection.

PRESERVING THE Tfh PROGRAM IN RESTING MEMORY Tfh CELLS

A complex combination of cytokines, transcription factors (52), STAT molecules (53), and epigenetic changes (54, 55) combine to delineate both the initial differentiation and stability of T helper lineages. Extrinsic factors that drive CD4 T cell activation and T helper cell differentiation are absent after antigen/pathogen clearance. Consequently, transcription factors such as Tbet and Bcl6 and other molecules that define T helper lineages are downregulated on memory CD4 T cells (20, 31, 40, 41). A critical question regarding the existence of CXCR5+ Tfh memory cells (and other specialized T helper cell memory subsets) remains: how do memory cells maintain lineage commitment and remember the gene expression programs of their previously defined T helper lineage in the absence of antigen and inflammatory signals (**Figure 4A**) (56, 57)? While the answer to this question for memory Tfh cells is currently not resolved, it is likely that the

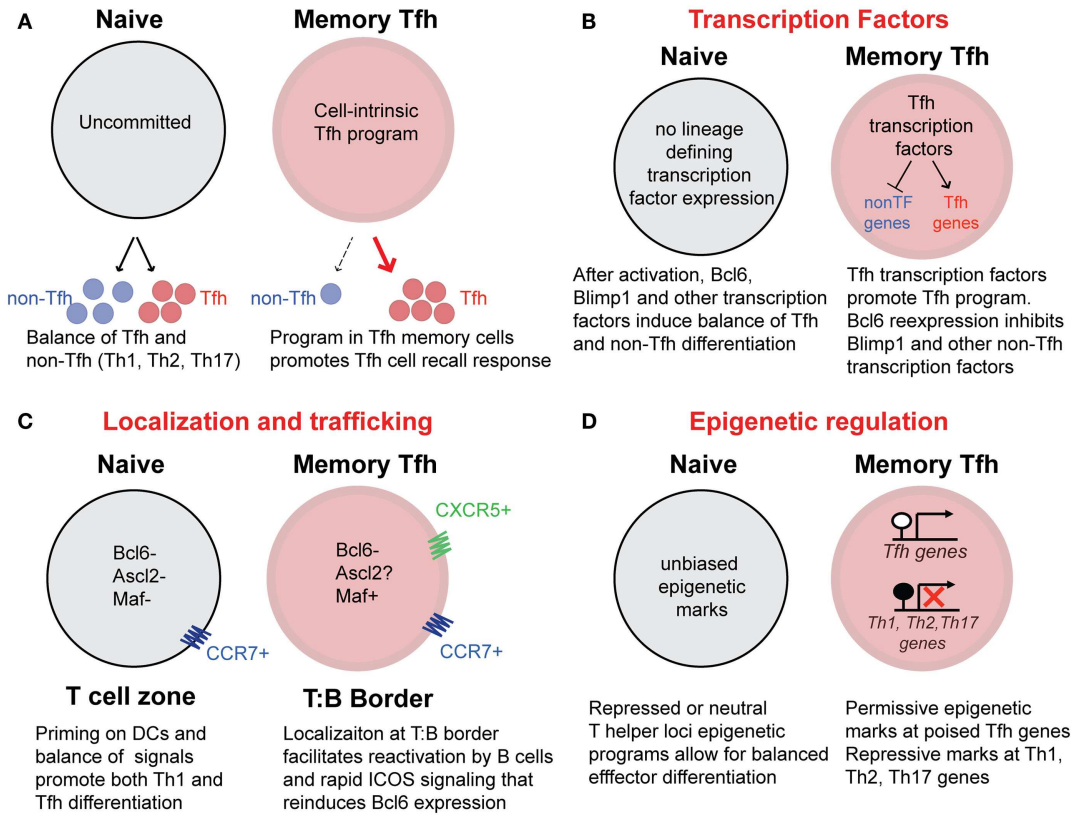

FIGURE 4 | Potential mechanisms that promote Tfh recall responses from memory Tfh cells. **(A)** Differentiated CXCR5+ memory Tfh cells possess cell-intrinsic programs that promote the preferential generation of Tfh secondary effectors cells upon reactivation with antigen. In contrast, uncommitted naïve CD4 T cells are pluripotent, with the capacity to generate a balance of Tfh and non-Tfh (Th1, Th2, Th17) effector cells following activation. **(B)** The maintenance of Tfh associated transcription factors may promote the recall of the Tfh cell program and inhibit the differentiation of other T helper lineages following activation. The transcription factor Maf, is maintained at low levels in human Tfh memory cells. While Bcl6 expression is not detectable in memory Tfh cells, it is possible that soon after reactivation, rapid reexpression of Bcl6 directs Tfh gene expression as well as inhibits Blimp1 and high expression of Tbet, Gata3, and RORγt. **(C)** CXCR5 and CCR7 coexpression on memory Tfh cells may provide localization of memory Tfh cells along the T:B cell border either before or early after reactivation, promoting increased interactions with B cells that can reinforce the Tfh cell phenotype and function. In contrast, CCR7+ (CXCR5−) naïve CD4 T cells will become activated in T cells zones by antigen-bearing dendritic cells. **(D)** Epigenetic programs acquired during Tfh effector differentiation can be maintained in resting memory Tfh cells throughout homeostatic proliferation. Transcriptionally permissive histone modifications and unmethylated DNA at "poised" Tfh associated loci in memory Tfh cells will promote the rapid reexpression of these genes upon reactivation. In addition, repressive histone modifications and DNA methylation will prevent reexpression of Th1, Th2, and Th17-associated genes.

reexpression of key transcription factors, as well as the epigenetic state of genes related to Tfh cell development and function, play an important role in directing Tfh memory cells toward recalling a secondary Tfh response. Potential mechanisms (that are not mutually exclusive – and more likely work in concert) for preserving the program of Tfh memory cells that promote the recall of Tfh secondary effector cells are shown in **Figures 4B–D**.

Bcl6 plays a critical role in defining the phenotype and function of Tfh cells (8–10). Although all of the mechanisms by which this transcriptional repressor positively directs the Tfh gene expression program, Bcl6 expression can induce CXCR5 expression (9, 15), likely through an indirect mechanism. Bcl6 overexpression in naïve human CD4 T cells promotes the regulation of genes required for germinal center trafficking and interactions with B cells, and in cooperation with Maf induces expression of other key Tfh genes (15). It is clear that while many Tfh associated genes are downregulated in resting CXCR5+ memory cells, these cells maintain a Tfh-like gene expression profile for many other Tfh associated genes (20, 47). Bcl6 transcript was not detectable in memory Tfh cells (20, 41, 42, 47). However, this does not rule out that very low levels of Bcl6 expression could bias the recall of memory Tfh cells early after cell reactivation by promoting the Tfh phenotype and inhibiting Blimp1 activity (**Figure 4B**). Furthermore, because memory B cells can present antigen to and rapidly induce Bcl6 expression in memory Tfh cells (58), the Bcl6-dependent Tfh transcriptional program may be robustly reinforced in memory Tfh cells upon reactivation *in vivo*.

Maf gene and protein expression were found at low levels in human blood Tfh memory cells (CXCR5+CXCR3−PD-1+) (47), suggesting that this important transcriptional regulator of Tfh cell functions (14, 15) may play a role in preserving aspects of the Tfh phenotype and program in these memory cells. In addition, Ascl2, a recently described transcription factor was shown to be required for early Tfh differentiation and function (16). Overexpression of Ascl2 results in CXCR5 expression, CCR7 downregulation, and subsequent migration to the follicles. Ascl2 binds to conserved non-coding sequence regions of the *Cxcr5* locus and promotes *Cxcr5* gene expression. Interestingly, Ascl2 did not induce Bcl6 expression. It is therefore possible that Ascl2 expression in Tfh memory cells may be important for maintenance of CXCR5 expression in resting memory Tfh cells (16). Currently, it is unclear what specific roles Bcl6, Maf, Ascl2, or other transcription factors play in preserving CXCR5 expression on memory Tfh cells, and promoting maintenance of the Tfh gene expression program (**Figure 4B**). Upon reactivation, it is possible that CXCR5+ Tfh memory cells are already localized (or rapidly relocalize) to the T:B border, resulting in interactions with B cells that would then reinforce Bcl6 reexpression and other Tfh transcription factors (**Figure 4C**). In turn, this might preferentially promote the Tfh program (over other T helper lineage programs) during the secondary response (**Figure 4C**). Currently, it remains unclear how these and other transcription factors influence the maintenance of the Tfh program and the repression of other T helper cell lineages in Tfh memory cells.

Transcription factors that have been termed lineage "master regulators," such as Tbet, RORγt, and Gata3 are not always limited in their expression to a single subset of differentiated CD4 T cells and can be expressed in Tfh effector cells (**Figure 1**) (1). The expression of these factors in Tfh effector cells is believed to be important for promoting expression of key cytokines that direct specific isotype class switching in cognate germinal center B cells (17, 21, 22). The balance of Bcl6 and Tbet has been shown to have important effects of differentiation and gene expression by Th1 and Tfh cells (59–61). Interestingly, following LCMV infection, mouse Tfh cells co-express Bcl6 and intermediate levels of Tbet (20). The resulting Tfh memory cells do not have detectable Bcl6, but surprisingly, maintain low levels of Tbet gene and protein expression (20). Despite this Tbet expression (and lack of Bcl6), adoptively transferred Tfh memory cells predominantly generate Tfh secondary effector cells following reinfection (20). This finding invokes the idea that beyond the simple balance of lineage-associated transcription factors, transcriptional programing through epigenetic modifications likely plays a role in promoting the reexpression of Tfh genes and repressing the gene expression programs of other T helper cell lineages (57).

The maintenance of gene expression and lineage-differentiation programs in the absence of inflammatory/differentiating signals is reinforced by epigenetic programs that are acquired during the initial effector T cell differentiation (54, 57). The combination of epigenetic modifications to histones to regulate the structure of chromatin and the methylation of DNA at CpG motifs that determine the binding of inhibitory methyl-binding domain proteins can be conserved in daughter cells throughout cell division (62), providing a mechanism for gene expression programs to be maintained during T cell homeostatic proliferation (57). Furthermore, transcriptionally permissive epigenetic marks are positively associated at loci of lineage-specific cytokines for each T helper lineage, while repressive marks at specific loci relevant to other T helper lineages serve to prevent inappropriate gene expression and suppress the differentiation toward alternative lineages (54, 57). Lu et al. observed that positive chromatin marks were associated in Tfh-like cells at the *Tbx21*, *Gata3*, and *Rorc* loci, and that these cells could be repolarized toward Th1, Th2, and Th17 cells when restimulated under polarizing conditions (63). This study highlights that certain features of the Tfh program may partially overlap with Th1, Th2, and Th17 cell programs, possibly allowing greater plasticity toward the capacity to produce multiple lineage-defining cytokines. In addition, *in vitro* differentiated Th1 and Th2 cells (but not Th17 cells) can become Tfh cells following adoptive transfer and immunization with protein in adjuvant (42). Together, these studies demonstrate that there is a degree of flexibility between effector Tfh cells and other T helper cells. Further work is required to determine whether and how such flexibility applies to CXCR5+ memory Tfh cells generated *in vivo*. It is possible that context-dependent selective processes during memory T cell development promote antigen-specific memory T cells that are either more or less committed to Tfh differentiation compared to their effector (precursor) counterparts (**Figure 3**).

Methylation of CpG motifs in regulatory regions of genomic DNA serve as a transcriptionally repressive mark through recruitment of methyl-binding domain proteins (62, 64). Loss of DNA methyltransferase activity has dramatic effects upon CD4 differentiation, resulting in loss of restriction of key lineage-associated cytokine and transcription factor genes (65–69). DNA bisulfite

sequencing analysis of LCMV-induced CD4 T cells revealed that the *Gzmb* locus (encoding granzyme B) became demethylated in Th1 but not Tfh effector cells. Furthermore, the maintenance of DNA methylation at the *Gzmb* locus in CXCR5+ memory Tfh cells was predictive of both their capacity to recall the Tfh effector phenotype upon reactivation with antigen *in vivo*, and also their inability to efficiently express granzyme B, even among the small fraction of Th1-like secondary effector cells that had downregulated CXCR5 expression (20). Thus, repression of Th1-associated gene expression programs (such as *Gzmb*) in memory Tfh cells likely plays an important role in the maintenance of the Tfh program in resting CXCR5+ Tfh memory cells by repressing the expression of genes associated with Th1 effector function (**Figure 4D**). Future work is needed to characterize permissive versus repressive epigenetic marks (for loci of lineage-associated cytokines, effector molecules, and transcription factors) in antigen-specific memory Tfh, Th1, Th2, and Th17 cells, providing needed insight into the plasticity versus commitment of memory CD4 T cell subsets induced by infection and immunization.

CONCLUDING REMARKS

The discovery that phenotypically unique subsets of CXCR5+ memory CD4 T cells have recall potential specific for Tfh function invites important questions for future study that will inform vaccination strategies for infectious diseases. Understanding the mechanisms of how Tfh memory cells acquire and preserve the Tfh gene expression programs and preferentially recall these programs upon reactivation will provide important insight into the lineage maintenance and plasticity of these cells. Finally, future work is needed to determine the optimal ways to utilize Tfh memory cells during prime and boost immunization to promote ways that improve protective and long-lived antibody responses.

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EPFL Innovation Park · Building I · 1015 Lausanne · Switzerland
T +41 21 510 17 00 · info@frontiersin.org · frontiersin.org