T CELL REGULATION BY THE ENVIRONMENT

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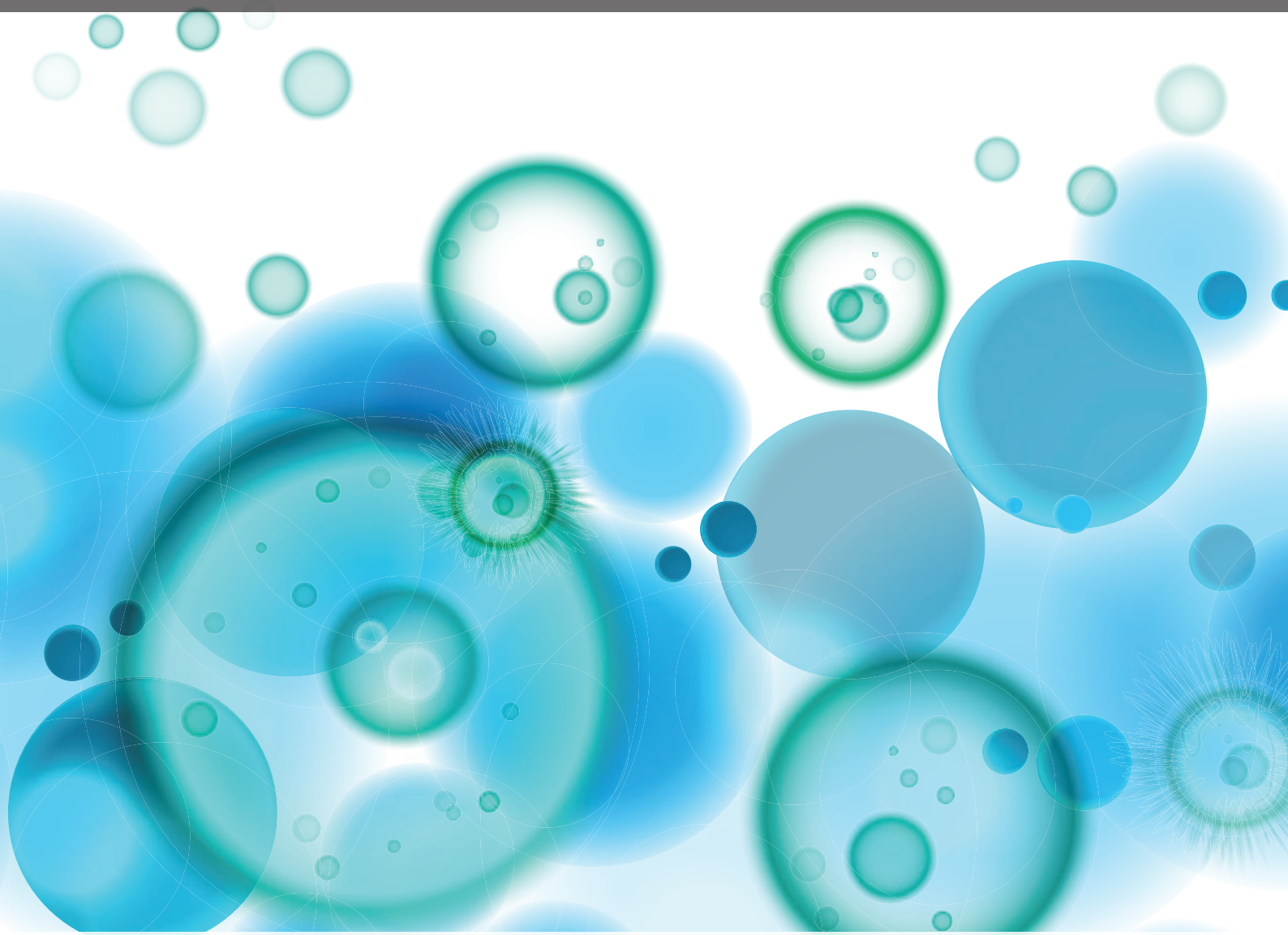

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T CELL REGULATION BY THE ENVIRONMENT

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Naïve T cells get activated upon encounter with their cognate antigen and differentiate into a specific subset of effector cells. These T cells are themselves plastic and are able to re-differentiate into another subset, changing both phenotype and function. Differentiation into a specific subset depends on the nature of the antigen and of the environmental milieu. Notably, certain nutrients, such as vitamins A and D, sodium chloride, have been shown to modulate T cell responses and influence T cell differentiation. Parasite infection can also skew Th differentiation. Similarly, the gut microbiota regulates the development of immune responses. Lastly, the key role of metabolism on T cells has also been demonstrated. This series of articles highlights some of the multiple links existing between environmental factors and T cell responses.

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EDITORIAL
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Editorial: T cell regulation by the environment

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Keywords: T cells, environment, metabolism, microbiome, vitamin D, regulatory T cells, pathogens

T cell responses are initiated by ligation of their cognate T cell receptor by MHC loaded with antigenic peptide, but their response is carefully controlled by a myriad of environmental cues, including co-activation receptors, cytokines, nutrients, growth factors, local oxygen levels, salt concentrations, and microbiome. The complexity of the integration of signals received by T cells is only beginning to be fully understood (1). This research topic in T cell biology aims at highlighting some of the latest research on intrinsic and extrinsic signals regulating T cell responses. The ebook contains 10 articles that encompass key pathways that modulate T cell function and discuss how T cells coordinate their response to environmental cues.

One of the first components regulating T cell activation is the expression and subsequent activation of surface receptors. Notably, T cell activation is governed by the co-activation of the TCR and of co-stimulatory or co-inhibitory molecules (2, 3). Expression and activation of co-inhibitory molecules, such as CTLA-4 and PD1 play a key part in turning off effector responses and the balance of expression of co-stimulatory and co-inhibitory receptors needs to be tightly regulated to ensure a proper level of T cell activation (4, 5). The review by Schneider and Rudd nicely illustrates how expression of the co-inhibitory molecule CTLA-4 is regulated, and how that affects T cell activation (6).

In recent years, the importance of cellular metabolism in the activation of immune cells, in particular T cells, has been reported. T cell activation requires a change in cellular metabolism to face increased energetic demand. This is an exciting area of research showing that not only changes in metabolism are necessary for cell activation but that they are also actively involved in regulating T cell function and differentiation. This is discussed in several reviews. Craig Byersdorfer focuses on the role of fatty acid oxidation for T cell functions, and notably on its role in graft versus host disease (7). Ramsay and Cantrell discuss the importance of glucose metabolism for T cell function and highlight the role of hypoxia-inducible factor alpha and mTOR in coordinating the responses to the environmental cues (8). They also summarize the importance of the microbiome in regulating T cells, and the key role of the aryl hydrocarbon receptor in sensing microbes. Clovis Palmer and collaborators review glucose metabolism in T cells and also discuss how HIV infection modulates T cell metabolism (9). One of the central questions is how do T cells integrate the multitude of signals received? In their comprehensive review, Chapman and Chi discuss the central role of mTOR in the integration of the many signals received by the T cells that ultimately shape their response (10).

Another related aspect of the topic is the impact of infection on T cells, whereby pathogens control the host response, mostly to their advantage, and are able to switch T cell responses to favor their own survival. A study by the group of Francisca Mutapi describes a novel mechanism by which helminth infection downregulates T cell activation, by lowering the level of CD3zeta chain in infected individuals (11). A review from Zaunders further illustrates how HIV infection affects the balance of the various T cell subsets and promotes regulatory T cells and T helper follicular cells (12).

Diet can also influence immune homeostasis, with recent studies showing, for instance, how salt can affect Th differentiation (13). The effects of vitamins and their metabolites on T cells are also well described. Colleen Hayes' group summarizes the latest data on the effects of Vitamin D on T cell

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responses, and how this can be modulated in autoimmune diseases (14). A review by Leving's group focuses more particularly on the environmental factors that affect regulatory T cells. This includes cytokines, vitamin A and vitamin D, metabolism, and microbiome (15).

Finally, can we therapeutically manipulate the environmental milieu to modulate T cell responses in humans? The study by the group of David Klatzmann highlights the effects of IL-2 in modulating T cell responses in type 1 diabetes (16). While the functions of regulatory CD4+ Tregs have been described years ago, the characterization of CD8+ Tregs is more recent. The authors notably report how injection of low doses of IL-2 modulates the levels of CD8+ Tregs *in vivo*, in both mice and in patients with type 1 diabetes, following their numbers and phenotype. This study illustrates how exogenous cytokines can modulate *in vivo* T cell responses, which may prove beneficial for autoimmune diseases.

We hope that this compilation of reviews and research that provides an overview on environmental regulators of T cells will give the readers a flavor on the latest development in T cell biology. We would also like to thank all the contributors to this topic and the reviewers for their time in making this ebook possible.

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REVIEW ARTICLE
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Diverse mechanisms regulate the surface expression of immunotherapeutic target CTLA-4

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T-cell co-receptor cytotoxic T-cell antigen-4 (CTLA-4) is a critical inhibitory regulator of T-cell immunity and antibody blockade of the co-receptor has been shown to be effective in tumor immunotherapy. Paradoxically, the majority of CTLA-4 is located in intracellular compartments from where it is transported to the cell surface and rapidly internalized. The intracellular trafficking pathways that control transport of the co-receptor to the cell surface ensures the appropriate balance of negative and positive signaling for a productive immune response with minimal autoimmune disorders. It will also influence the degree of inhibition and the potency of antibody checkpoint blockade in cancer immunotherapy. Current evidence indicates that the mechanisms of CTLA-4 transport to the cell surface and its residency are multifactorial involving a combination of immune cell-specific adapters such as TRIM and LAX, the small GTPase Rab8 as well as generic components such as ARF-1, phospholipase D, and the heterotetrameric AP1/2 complex. This review covers the recent developments in our understanding of the processes that control the expression of this important co-inhibitory receptor for the modulation of T-cell immunity. Interference with the processes that regulate CTLA-4 surface expression could provide an alternate therapeutic approach in the treatment of cancer and autoimmunity.

Keywords: CTLA-4, trafficking, TRIM, LAX, Rab8

INTRODUCTION

The co-receptor cytotoxic T lymphocyte antigen-4 (CTLA-4; CD152) is a central inhibitory regulator of T-cell proliferation and expansion (1–5). Its dampening effect on the activation process limits and terminates T-cell responses, and as such is important for regulating peripheral T-cell tolerance and autoimmunity. A negative role for the co-receptor in the control of proliferation and autoimmunity was initially observed in the striking phenotype of the *Ctla4−/−* mouse (6, 7). These mice show polyclonal T-cell activation or autoproliferation that leads to massive tissue infiltration and early lethality. An additional linkage of single-nucleotide polymorphisms (SNPs) in the region of CTLA-4 were subsequently found associated with a variety of autoimmune disorders that include type 1 diabetes, coeliac disease, myasthenia gravis, Hashimoto's thyroiditis, systemic lupus erythematosus (SLE), and Wegener's granulomatosis (8–12). Immune dysregulation in human subjects has also been reported recently with heterozygous germline mutations in CTLA-4 (13). This plurality of associated autoimmune disorders in human beings has pointed to a central role for the co-inhibitory receptor as a general regulator of the threshold signals needed for T-cell activation. Under normal conditions, the inhibition of signaling events protects against responses to lower affinity self-antigen while allowing responses to higher affinity foreign antigen. In this sense, minor changes in the surface expression of the co-receptor are thought to have significant effects on responses to autoantigen. Ipilimumab, a humanized anti-CTLA-4 checkpoint blockade antibody, has also been found impressively effective in the treatment of various tumors such as melanoma and small cell lung carcinomas (14, 15). Combined therapy with antibodies against another negative co-receptor PD-1 (programmed cell death-1) has been found to co-operate with anti-CTLA-4 to induce even more striking response rates (16).

Given that minor changes in the surface expression of the co-receptor are expected to have significant effects on responses to autoantigen and in cancer immunotherapy, it is important to understand the mechanisms that determine the expression of CTLA-4 on T-cells. This includes the intracellular pathways that determine the transport or trafficking of CTLA-4 to the cell surface as well as events that regulate its residency on the surface and endocytosis. Paradoxically, CTLA-4 is primarily located in intracellular compartments from where it is rapidly recycled to the cell surface. Only small amounts of the co-receptor can be detected on the cell surface at any given time, even when optimally expressed following T-cell activation. This review covers the recent developments in our understanding of the events that control the transport and expression of CTLA-4 to the cell surface for the modulation of T-cell immunity.

STRUCTURE AND FUNCTION OF CTLA-4

CTLA-4 was one of the first and most extensively investigated co-inhibitory receptor of the immune system (17). The CTLA-4 gene consists of four exons: exon 1 contains the leader peptide sequence, exon 2 the ligand binding site, exon 3 encodes the transmembrane region, and exon 4 the cytoplasmic tail (18). Differential splicing of the CTLA-4 transcript results in a full-length transmembrane form (exons 1–4), soluble CTLA-4 (lacking exon 3), and a transcript encoding only for exons 1 and 4 (19,

20). Murine T-cells also express a ligand-independent CTLA-4 (liCTLA-4) containing exons 1, 3, and 4 (12). Although liCTLA-4 lacks the MYPPPY ligand binding domain, it strongly inhibits T-cell responses and, compared to full-length CTLA-4, its expression is elevated in regulatory and memory T-cells from diabetes resistant NOD mice (21).

CTLA-4 is structurally related to CD28 with some 30% sequence homology (22). It was first described as the product of the *Ctla4* gene located at chromosome 1 (mouse) or 2 (human being) and is preferentially expressed in activated cytolytic T-cells (17). Subsequently, it was found to be expressed in all activated T-cells and used as an early activation marker. mRNA for CTLA-4 can be detected as early as 1 h post-activation with maximum expression between 24 and 36 h, the time when CTLA-4 is detectable on the cell surface (23, 24). In contrast to full-length CTLA-4, ligand-independent CTLA-4 is expressed in resting cells, but downregulated during early activation (21). Like CD28, CTLA-4 binds to ligands CD80 and CD86 but with greater avidity (25, 26). The same signature MYPPPY motif for binding is found in both co-receptors (27). The higher avidity of CTLA-4 for CD80 is due to the binding of one CTLA-4 homodimer to two CD80 molecules (28, 29) resulting in the formation of a stable CTLA-4/CD80 lattice structure in the immunological synapse (IS). This interaction may disturb the assembly of key signaling proteins needed for CD28 co-stimulation.

As mentioned, the importance of CTLA-4 in maintaining peripheral tolerance and homeostasis was first demonstrated with the autoimmune phenotype of CTLA-4-deficient mice. These mice show polyclonal T-cell activation leading to massive tissue infiltration and early lethality (6, 7). Further, SNPs of the human CTLA-4 gene have been implicated in the susceptibility to autoimmune disorders such as type I diabetes, rheumatoid arthritis, and multiple sclerosis (12). However, it is still unknown how and whether SNPs affect CTLA-4 function (i.e., intracellular trafficking, surface expression, dimerization). The soluble form of CTLA-4 has been linked to autoimmune diseases. High concentrations of soluble CTLA-4 can be detected in patients with various autoimmune diseases (30–32).

Unlike in the case of conventional T-cells (Tconv), suppressive regulatory T-cells (Tregs) express CTLA-4 constitutively on the cell surface. In fact, the pool of intracellular CTLA-4 seen in activated Tconv is less apparent in Tregs, a finding that may account for its constitutively high level of surface expression (33). Given this fact, it is not surprising that CTLA-4 is intimately linked to the regulation of Treg suppressor function (34, 35). Mechanisms that have been reported to account for Treg function include the secretion of the suppressive cytokines IL-10, IL-35, and TGF-β (36), secretion of cytolytic granules containing granzyme and perforin as well as competition with conventional responder T-cells for CD80 and CD86 on antigen-presenting cells (APCs) (37, 38). Given its higher avidity for binding to CD80/86, CTLA-4 would block the availability of CD80 and CD86 for an interaction with Tconv. While CTLA-4 on Tconv induces their motility and limits their contact time with APCs, resulting in hypoactivation of these cells, CTLA-4 on Tregs does not influence their dwell times and, therefore, would allow the co-receptor to interfere with CD80/86 presentation to CD28 (39).

CTLA-4 AND TUMOR IMMUNOTHERAPY

An exciting development over the past few years has been the use of anti-CTLA-4 in so-called checkpoint blockade in the treatment of cancers. These human studies originated from earlier mouse tumor models, which demonstrate that blockade of CTLA-4-mediated inhibition leads to enhancement of T-cell responses in tumor immunotherapy (40). Early human studies with limited numbers of patients (41–44) were expanded to larger phase III studies showing response rates as high as 30% on melanoma, small cell lung, and renal carcinoma (14–16). These studies led to the generation of antibodies to human CTLA-4, ipilimumab, and tremelimumab (45). Ipilimumab has been approved as monotherapy for the treatment of advanced melanoma. They have shown synergistic anti-tumor activity when utilized with vaccines, chemotherapy, and radiation (14). CTLA-4 antibodies have also induced a reversible occurrence of immune-related adverse events (IRAE) such as colitis, dermatitis, or endocrinopathies (46). The exact mechanism by which anti-CTLA-4 mediates enhanced anti-tumor reactivity is not clear, but may involve a combination of effects involving the lowering of the threshold needed to activate T-cells, a reduction in the number of Tregs, the reduced release of the suppressive factor indoleamine 2,3-dioxygenase (IDO) as well as broadening the peripheral T-cell receptor repertoire (47, 48). In certain instances, co-operation with interleukin-2 treatment has also been observed (49). More recently, antibodies against PD-1, another inhibitory co-receptor, have also demonstrated remarkable clinical anti-tumor activity against melanoma and other solid tumors (50). Further, the combination of anti-CTLA-4 and PD-1 antibodies achieved an even more effective anti-tumor response (16, 51). CTLA-4 engagement with CD80/CD86 attenuates the early activation of naïve and memory T-cell, whereas PD-1 is mainly thought to modulate T-cell effector functions in peripheral tissues via binding to PD-L1 and PD-L2 (52). Since CTLA-4 and PD-1 regulate immune responses in a non-redundant fashion, combined blockade of both pathways may achieve more effective anti-tumor activity.

MECHANISMS OF CTLA-4-MEDIATED INHIBITION

Despite the importance of CTLA-4 to autoimmunity and anti-tumor immunotherapy, the actual mechanisms responsible for its function are unknown. Much debate has focused on whether CTLA-4 inhibits T-cell responses by cell-extrinsic or -intrinsic mechanisms. Cell intrinsic mechanisms would reflect direct effects of the co-receptor on the expressing cell (i.e., signal transduction), while cell-extrinsic effects relate to the regulation of function via a distal cell or cytokine. Both mechanisms have been implicated in the *in vivo* function of CTLA-4 (53). A cell-extrinsic pathway for CTLA-4 was first described by Bachman and coworkers who found that Rag2-deficient mice reconstituted with a mixture of wild-type and CTLA-4-deficient bone marrow cells failed to develop autoimmune disease, while the transfer of *Ctla4*−/− bone marrow cells alone transferred disease (54). Cell-intrinsic and non-cell-autonomous (i.e., cell extrinsic) actions of CTLA-4 have been reported to operate to maintain T-cell tolerance to self-antigen (53). In agreement with this observation, Thompson and coworkers found that the loss of the cytoplasmic tail of CTLA-4 (i.e., cell intrinsic) affected the onset of disease as well as differences

in T-cell infiltration. These findings suggested possible differences for cell intrinsic versus extrinsic mechanisms in the autoproliferative versus migratory aspects of CTLA-4 inhibition (55). Others have emphasized the importance of cell-extrinsic mechanisms on both Tconv and Tregs, although this may vary with antigen dose and the model examined (56). It is possible that CTLA-4 utilizes different pathways for inhibition in different contexts or niches of the immune system.

Cell intrinsic pathways include modulation of TCR signaling by phosphatases SHP-2 and PP2A (57), inhibition of ZAP-70 microcluster formation (58), and altered IS formation (59), as well as interference with the expression or composition of lipid rafts on the surface of T-cells (60–63). Like CD28 and ICOS, CTLA-4 possesses a small cytoplasmic tail containing, apart from its C-terminal YFIP motif, a YxxM consensus motif common of all three co-receptors (64) (**Figure 1**). Several intracellular proteins including the lipid kinase phosphatidylinositol 3-kinase (PI3K) (65), the phosphatase SHP-2 (4, 57, 66, 67) and clathrin adapter proteins AP1 and AP2 (68–70) have been reported to bind to the YVKM motif. The phosphatase PP2A has also been reported to interact with the cytoplasmic tail of CTLA-4 via the lysine rich motif and via the tyrosine residue at position 218 (71). CTLA-4-mediated phoshorylation of Akt is abrogated by the PP2A inhibitor okadaic acid (72). By contrast, PD-1 signaling inhibits Akt phosphorylation by preventing CD28-mediated activation of PI3K that is dependent on the immunoreceptor tyrosine-based switch motif (ITSM) located in its cytoplasmic tail (72).

Cell-extrinsic mechanisms include CTLA-4 engagement of CD80/CD86 on dendritic cells (DCs) that can induce the release of IDO (73, 74). This enzyme catalyzes the degradation of the amino acid L-tryptophan to *N*-formylkynurenine leading to the depletion of tryptophan, which in turn can halt the growth of T-cells. Although IDO has been implicated in certain immune responses (75, 76), it is unlikely to solely account for the phenotype of the *Ctla4*−/− mouse since IDO-deficient mice fail to develop autoimmunity (77). CTLA-4 has also been reported to increase the production of the immunosuppressive cytokine TGF-β (78); however, TGF-β-deficient mice differ from CTLA-4-deficient mice in the severity of the autoimmune phenotype (79). The multiorgan inflammatory syndrome can be inhibited by depletion of the activated CD4 positive T-cells leading to prolonged survival; however, the TGF-β-deficient mice eventually die of myeloid hyperplasia (80).

Not unexpectedly, Tregs play a major role in cell-extrinsic regulation. Both CTLA-4-deficient and FoxP3-deficient mice exhibit a short life span due to massive lymphoproliferation (LP) and a systemic autoimmune-like syndrome (6, 7, 81). The conditional loss of CTLA-4 on FoxP3 expressing cells delayed the onset of disease to 7–10 weeks, rather than to 3–4 weeks observed in *Ctla4*−/− mice (82, 83). This indicated that Tregs help control the development of the *Ctla4*−/− phenotype and that both CTLA-4 and FoxP3 on the same cell subset are essential to fully prevent LP disease. However, while Tregs help to control the onset of disease, the fact that the mice still die suggests that another factor is causally responsible for the onset of the autoimmune-like syndrome.

The mechanism by which CTLA-4 facilitates Treg function is unclear but may involve the occupancy of CD80 and CD86 on DCs (82, 83). Trans-endocytosis or the removal of CD80 or CD86 from the surface of DCs may also occur (83, 84). Since both Tregs and Tconv can mediate this removal, it is uncertain whether this property can be the primary mechanism to account for Treg suppression. On the other hand, in certain models, some groups have claimed that the mere expression of CTLA-4 on either subset is sufficient to mediate cell-extrinsic suppression (33, 56). Tregs with higher CTLA-4 levels are able to be more effective in blocking or trans-endocytosis than Tconv cells. In this context, recent elegant work has shown that CTLA-4 can bind to the protein kinase C isoform η (PKC-η) in Tregs (and not Tconv cells) and that defective activation of CTLA-4-PKC-η with another complex in PKC-η-deficient cells correlates with the reduced depletion of

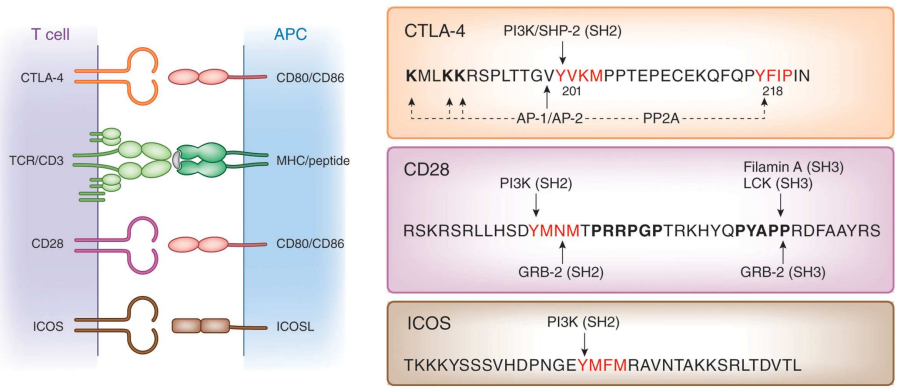

FIGURE 1 | Structure of co-receptors. Left panel: CTLA-4 and CD28 bind to the same natural ligands CD80/CD86 via the MYPPPY motif, whereas ICOS binds to ICOSL via the FDPPPF motif. **Right panel**: structure of the cytoplasmic domains of human CTLA-4, CD28, and ICOS. The cytoplasmic domains of these co-receptors have a common YxxM motif, which binds to the SH2 domain of the p85 subunit of phosphatidylinositol 3-kinase (PI3K). CTLA-4 has a unique YVKM motif, which binds to the SH2 domain of the tyrosine phosphatase SHP-2. In its non-phosphorylated form, it associates with the clathrin adapters AP-1 and AP-2. The serine/threonine phosphatase PP2A binds to the lysine rich motif and the tyrosine 218 (Y_{218}FIP). The asparagine in the YMNM motif of CD28 is needed for Grb-2 SH2 domain binding, whereas the distal proline motif allows for binding of the SH3 domains of Grb-2, the protein tyrosine kinase p56lck, and Filamin A.

CD86 from APCs (85). CTLA-4-associated SHP-1/2 and PP2A are not recruited to the IS of Tregs (85, 86).

Another model involves a combination of cell-intrinsic and -extrinsic effects related to altered T-cell adhesion and motility (87, 88). We and others have shown that CTLA-4 ligation activates the small GTPase Rap-1 (89, 90). Rap1 is a key molecule involved in the activation of integrins such as lymphocyte function-associated antigen-1 (LFA-1). In this model, CTLA-4 is a motility activator and augments T-cells adhesion (88, 90). Significantly, anti-CTLA-4 alone was able to induce motility of primary T-cells and cell lines (58, 88). As a motility activator, CTLA-4 bypasses the TCR-mediated stop-signal that is needed for stable interactions between T-cells and APCs. This provided an alternate mechanism to account for the dampening effect of CTLA-4 on T-cell activation and has been confirmed in several different models (87, 88, 90–95). In this model, the cell intrinsic pathway involves activation of Rap1 and the ligation efficiency of the TCR on Tconvs, while the cell-extrinsic pathway involves the regulation of T-cell binding to APCs. The reversal of the stop-signal by CTLA-4 was exclusively seen on Tconv and not Tregs (39).

CTLA-4 TRAFFICKING FROM THE *TRANS*-GOLGI NETWORK TO THE CELL SURFACE

Understanding the mechanisms by which CTLA-4 is transported to the cell surface will be the key to the development of novel strategies to increase or decrease its expression and functional effects. An ability to interfere with the trafficking pathways in T-cells would provide an alternate approach to the use of biologics such as anti-CTLA-4 antibodies. Previous studies have demonstrated the need of calcium for the release of CTLA-4 from the *Trans*-Golgi network (TGN) to the cell surface (69, 96), while other studies have implicated more generic processes involving the GTPase ADP ribolysation factor-1 (ARF-1) and phospholipase D (PLD) (97). However, these pathways are also involved in the transport of other non-lymphoid receptors and thus are not specific for CTLA-4. In this context, it has been demonstrated that TCRzeta (TCRζ) plays a central role in transporting the TCR to the cell surface (98, 99). TCRζ is a member of the type III transmembrane adapter proteins (TRAPs), which possess a short extracellular domain, a single transmembrane domain, and a relatively long cytoplasmic tail with several tyrosine phosphorylation sites (100, 101) (**Figure 2**). Based on the TCRzeta model, we hypothesized that this family of transmembrane proteins might play a general role in the transport of surface receptors. Other members of the TRAP family include TRIM (T-cell receptor-interacting molecule), LAX (linker for activation of X cells), SIT (SHP2 interacting TRAP), and LAT (linker for activation of T-cells) (100, 101). As in the case of the TCRζ, they are preferentially expressed in immune cells, but most of them lack the signaling effects seen with the TCRzeta chain. For example, they lack the immunoreceptor tyrosine-based activation motifs (ITAMs) needed for binding to the protein tyrosine kinase ZAP-70. Instead, they are enriched in binding sites for PI-3K and Grb-2/Gads (102, 103).

TRIM is highly expressed in thymocytes and CD4 positive T-cells and forms a disulfide-linked homodimer (104). It possesses three tyrosine-based motifs in its cytoplasmic tail (two YxxL motifs and one YxxM motif), where the YxxM motif binds to the p85 subunit of PI3 kinase (102) (**Figure 2**). Initial TRIM overexpression studies in Jurkat T-cells suggested that TRIM upregulates the surface expression of the TCR and mediates increased calcium release after TCR ligation (105). However, T-cell development, TCR surface expression, and signaling events induced by TCR ligation are not impaired in TRIM-deficient mice (104). LAX is expressed as a monomer and possesses a longer cytoplasmic tail (398 aa versus 186 aa), which contains eight tyrosine-based motifs; five of them represent binding sites for Grb-2/Gads (103). LAX negatively impairs TCR signaling events as shown with LAX overexpression studies in Jurkat T-cells leading to inhibition of p38 and NFAT/AP-1 (106). Although LAX deficiency does not impair lymphocyte development, T- and B-cells are hyperresponsive upon TCR or BCR ligation and show increased cell survival (107). Mutation studies of the tyrosine-based motifs revealed the importance of the binding signaling proteins (Grb-2, Gads, and PI3K) in the inhibitory function of LAX (103).

Initial shRNA knockdown and overexpression studies demonstrated that TRIM facilitates the transport of CTLA-4 to the cell surface (108, 109). TRIM specifically co-precipitated CTLA-4, but not other T-cell co-receptors such as CD28. Overexpression of TRIM potentiated CTLA-4 expression due to increased release to the cell surface, which in turn led to increased suppression of T-cell activation. Subsequently, LAX was also found to bind, co-localize,

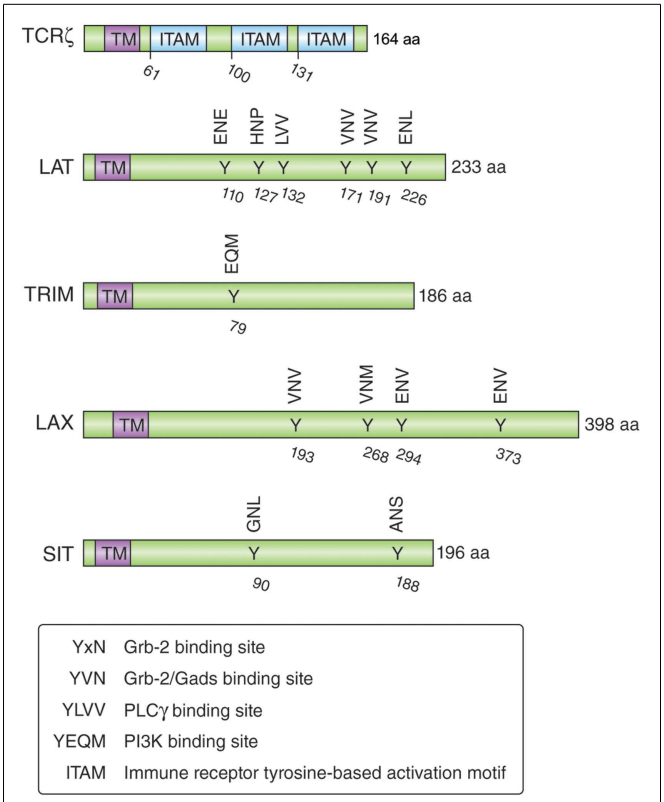

FIGURE 2 | Schematic structure of the transmembrane adapters TCRζ, LAT, TRIM, LAX, and SIT with their binding motifs for Grb-2, Gads, PI3K, and PLCγ.

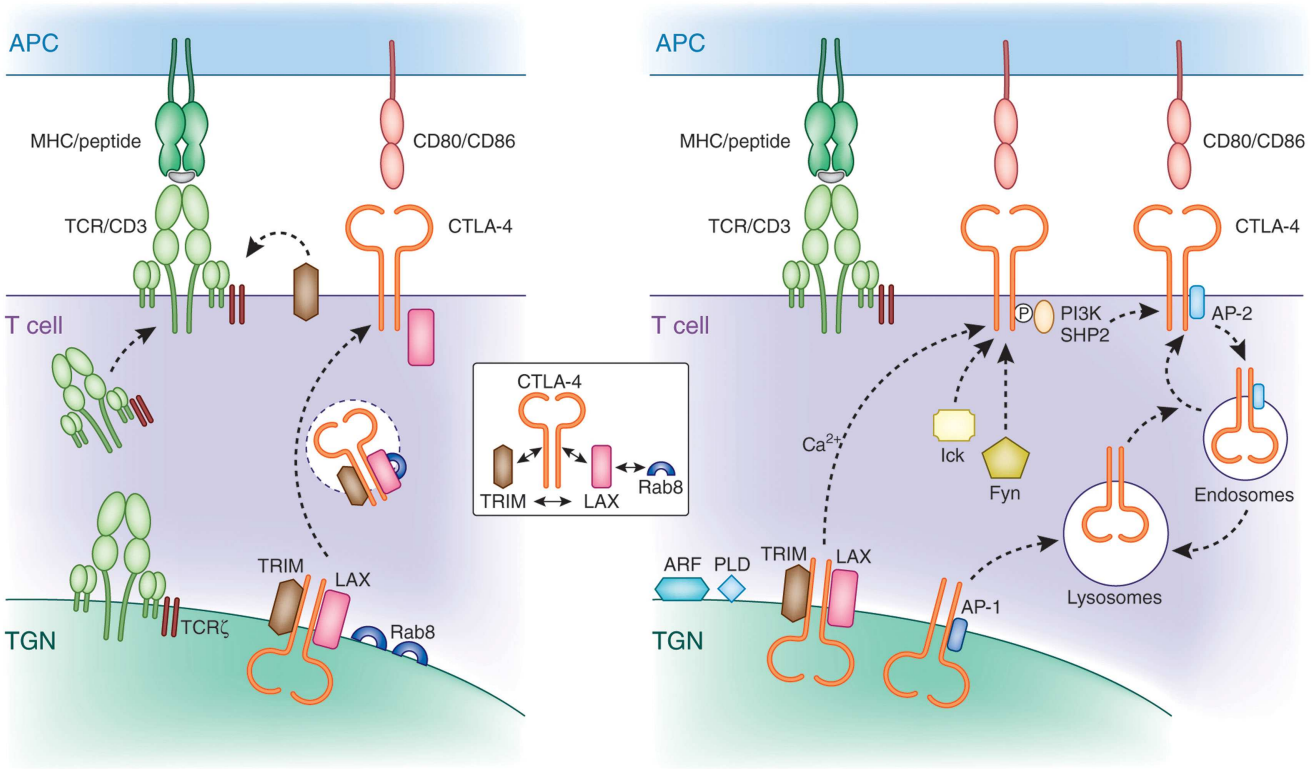

FIGURE 3 | Mechanisms of CTLA-4 trafficking. Left panel: CTLA-4 forms a multimeric complex composed of TRIM, LAX, and Rab8 for post-Golgi transport to the cell surface. TRIM and LAX bind to the cytoplasmic tail of CTLA-4, while LAX binds via its N-terminus to active GTP-Rab8 (inset). TRIM requires LAX for binding to Rab8 in a complex. LAX functions as a central coordinator by bridging Rab8 with the other LAX-associated proteins TRIM and CTLA-4. This multimeric complex facilitates the transport of newly translated CTLA-4 to the cell surface. The transmembrane adapters TRIM and LAX play a role for CTLA-4 transport similar to the TCRzeta chain, which resides in the TGN and surrounding vesicles and is needed for the association with the hexameric TCR/CD3 in the TGN for proper and efficient cell surface expression. **Right panel**: non-phosphorylated CTLA-4 associates with the clathrin adapter complex AP-1 and AP-2. AP-1 regulates trafficking of CTLA-4 by shuttling the receptor from the TGN to lysosomes. In activated cells, newly synthesized CTLA-4 is transported to the cell surface, which is facilitated by the TRIM/LAX/Rab8 complex (left panel) and by more generic factors such as ARF-1, PLD, and calcium. Phosphorylation of CTLA-4 by Fyn and Lck recruits PI3K and SHP-2. Dephosphorylated CTLA-4 binds to AP-2 leading to the internalization of CTLA-4 to intracellular compartments such as endosomes and lysosomes from where it can recycle to the cell surface.

and facilitate CTLA-4 transport to the cell surface (110). CTLA-4 binding to TRIM and LAX was specific in that it did not associate with LAT. These data indicate that TRIM and LAX, both immune-specific type III proteins, bind to CTLA-4 to facilitate its transport to the cell surface (**Figure 3**).

Strikingly, downregulation of TRIM and LAX markedly reduced the presence of CTLA-4 expressing TGN proximal vesicles. This observation suggested a connection between CTLA-4 binding to TRIM/LAX and the budding of CTLA-4 positive vesicles from the TGN needed for transport to the cell surface. Further, these findings resemble that found for the transport of the TCR/CD3 complex to the cell surface (111). TCRζ, which resides in the TGN and surrounding vesicles needs to associate with the hexameric TCR/CD3 in the TGN for proper and efficient cell surface expression. Rab proteins are members of the Ras superfamily and regulate protein transport of the secretory and endocytic pathway (112, 113). They are active in a GTP-bound state and become inactive with the conversion of GTP to GDP. Among the different members of the Rab family, Rab8 has been shown to mediate the trafficking of newly synthesized proteins from the TGN to the plasma membrane (114), whereas other family members mediate transport of proteins among other intracellular organelles such as endoplasmatic reticulum, endosomes, and lysosomes. In addition, Rab8 alters the reorganization of actin and microtubules, as well as directing membrane transport to cell surfaces (115, 116). Activation of Rab8 can lead to cell protrusions, whereas its depletion promotes the formation of actin stress fibers (117, 118). The $\alpha_2\beta$ and β_2-adrenergic receptors have been described to bind Rab8 for transport to the plasma membrane (119). However, until recently, despite its high expression in T-cells, no immune cell-specific binding effectors of Rab8 have been identified.

A recent study by Banton et al. showed that the transmembrane adaptor LAX bound to the active form of Rab8, while at the same time also associated with CTLA-4 and TRIM (110). By contrast, CTLA-4 and TRIM failed to bind to Rab8. These findings demonstrate that CTLA-4 interacts with a protein complex in which TRIM and LAX bind to the co-receptor, TRIM

and LAX to each other, and LAX to Rab8 (**Figure 3**, see inset). Importantly, disruption of LAX-Rab8 binding profoundly reduced the formation of CTLA-4 containing vesicles proximal of the TGN as well as the expression of CTLA-4 on the cell surface. The reduction in CTLA-4 expression resulted in augmented immune responses. Overall, given the established role of Rab8 as a molecule that mediates intracellular trafficking of proteins from the TGN to the plasma membrane, its association with CTLA-4 provides a pathway for the control of CTLA-4 surface expression.

Altogether, the TRIM-LAX-Rab8 connection to CTLA-4 trafficking to the cell surface will operate in co-operation with other generic processes. The ADP ribosylation factor (ARF) family GTPases and PLD are needed for the budding of vesicles at the Golgi apparatus (120–122). As in the case of Rab8, ARFs are members of GTP binding proteins of the Ras superfamily. There are six conserved ARF proteins in mammalian cells and are well established regulators of vesicle trafficking and actin re-modeling. In particular, ARF1 is involved in the regulation of vesicle transport in the TGN and the activation of PLD. PLD hydrolyzes phosphatidylcholine generating phosphatidic acid (PA) and choline. Further, the hydrolyzation of PA generates diacylglycerol, which, in addition to its signaling role, has a functional role in membrane modulation (123, 124). Dominant negative mutants of ARF-1 or PLD inhibit the release of CTLA-4 to the cell surface (97). However, unlike Rab8, none have been reported to associate with CTLA-4. Most likely, other key regulators of trafficking (i.e., SNAREs) are also needed for CTLA-4 expression. These mechanisms have been described for many surface expressed receptors and are not specific for CTLA-4.

RECYCLING OF CTLA-4

Following cell surface expression, CTLA-4 is rapidly internalized and again recycled to the plasma membrane of T-cells. This presumably occurs due to control of the inhibitory effects of CTLA-4 on the immune response (i.e., needs to be tightly regulated). The one exemption is Tregs, which show constitutively surface expressed CTLA-4 (24, 125). Rapid removal of CTLA-4 from the cell surface is mediated by the heterotetrameric adapter protein AP2 via clathrin-dependent internalization (68–70). AP-2 binding is regulated by the phosphorylation of the YVKM motif in the cytoplasmic tail of CTLA-4 (126, 127). Phosphorylation of CTLA-4 by protein tyrosine kinases p56lck, p59fyn, and Rlk (128, 129) promotes binding to PI3K or SHP-2 leading to the production of phosphatidylinositol (3,4,5)-triphosphate (D3 lipids) or dephosphorylation of tyrosine residues on substrates such as ITAMs in the TCR/CD3 complex and ZAP-70 (130). AP-2 binding to CTLA-4 is inhibited by the phosphorylation of the YVKM motif. Instead, once the recruitment and engagement of PI 3K is complete, CTLA-4 is dephosphorylated exposing the non-phosphorylated TGVYVKM motif. Binding of AP-2 generally involves the independently folded appendage domains of the large α (α_1 or α_2 isoform) and β_2 subunits, each separated from the heterotetrameric adapter core by a flexible hinge (131). Its binding to CTLA-4 mediates the internalization of the co-receptor from the cell surface to endosomal and lysosomal compartments. Golgi-associated CTLA-4 also binds to the heterotetrameric AP-1 via the same motif (69) where it shuttles the receptor from the TGN to lysosomes. Further, upon TCR stimulation, CTLA-4 containing secretory lysosomal vesicles are released to the cell membrane resulting in increased CTLA-4 surface expression (132). Further, under conditions of T-cell inactivation, CTLA-4 colocalizes with the TCR to lipid rafts in the IS (61). The polarized release of CTLA-4 toward the site of TCR engagement has been correlated with a repositioning of the microtubule organizing center (MTOC) in T-cells (96, 133). The extent of CTLA-4 surface expression is determined by the strength of the TCR signal (133). In contrast to full-length CTLA-4, ligand-independent CTLA-4 (lacking exon 2 encoding the ectodomain including the MYPPPY motif needed for CD80/86 binding) expressed in resting mouse T-cells is downregulated during activation (21). Also, compared to activated effector T-cells, CTLA-4 is considerably longer retained on the surface of memory T-cells (134). The molecular basis for the different expression levels of CTLA-4 in both cell types remains to be established. Intracellular trafficking to the cell surface as well as endocytosis and recycling determine the overall level of CTLA-4 on the surface of T-cells.

SUMMARY

Optimal regulation of CTLA-4 surface expression is crucial for the balance of stimulatory and inhibitory signals to elicit proper immune responses. Minor changes in surface expression levels could have major effects on the outcome of T-cell activation. Levels of surface expressed CTLA-4 are regulated by endocytosis, recycling, and newly synthesized CTLA-4. Besides generic factors (i.e., ARF-1, PLD, SNAREs) needed for transport of multiple receptors to the cell surface, the recently identified CTLA-4-TRIM-LAX-Rab8 complex is specific in facilitating CTLA-4 transport to the cell surface. This finding is of potential importance for the development of new therapeutics that will be designed to enhance anti-tumor immunity or to increase expression in the control of autoimmune disease. Cell permeable peptides (CPP) and/or siRNA targets of immune cell trafficking adapters TRIM or LAX could provide an alternate therapy especially for patients with severe IRAE during treatment with CTLA-4 antibodies. Further, a combination of anti-PD-1 antibodies and CPP could achieve an even more effective anti-tumor response.

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Environmental and metabolic sensors that control T cell biology

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The T lymphocyte response to pathogens is shaped by the microenvironment. Environmental sensors in T cells include the nutrient-sensing serine/threonine kinases, adenosine monophosphate-activated protein kinase and mammalian target of rapamycin complex 1. Other environmental sensors are transcription factors such as hypoxia-inducible factor-1 and the aryl hydrocarbon receptor. The present review explores the molecular basis for the impact of environmental signals on the differentiation of conventional T cell receptor αβ T cells and how the T cell response to immune stimuli can coordinate the T cell response to environmental cues.

Keywords: T cell metabolism, glucose uptake, hypoxia, amino acid uptake, leucine and mTOR, aryl hydrocarbon, microbiome

INTRODUCTION

T lymphocytes respond to immune stimulation by clonally expanding and differentiating to effector cells that produce the cytokines, chemokines, and cytolytic molecules that mediate adaptive immune responses. This process of T cell differentiation requires the cells to reprogram metabolism to meet the demands caused by the increases in macromolecule biosynthesis that accompany T cell activation (1–4). Naïve peripheral T cells are thus small cells that are metabolically quiescent with low rates of nutrient uptake and protein synthesis. Immune-activated T cells up-regulate glucose, amino acid, and iron uptake in a response that increases cellular energy production to support the biosynthesis of the proteins, DNA, and lipids necessary for cell growth and clonal expansion. Regulation of nutrient uptake is necessary for the differentiation of both naïve CD4 and CD8 T cells into effector and memory sub populations (5–7).

Accordingly, there is the potential for the T cell immune response to be shaped by the T cell nutrient microenvironment. In this context, it is increasingly recognized that antigen and cytokine stimuli coordinate how T cells respond to environmental cues by controlling the expression of nutrient receptors on the T cell membrane and by controlling the expression and function of environmental-sensing transcription factors that control T cell differentiation (5, 8–10) (**Figure 1**). The present review will explore some recent advances in our understanding of how environmental signals can impact on the differentiation of conventional T cell receptor (TCR)αβ T cells and how the T cell response to immune stimuli can coordinate the T cell response to environmental cues. Specifically, we will review glucose, oxygen, amino acid, and microbiome sensors and their impact on T lymphocyte biology.

GLUCOSE SENSORS AND T CELLS

Naïve and memory T cells have very low rates of glucose uptake and predominantly produce ATP via oxidative phosphorylation. T cell activation during an adaptive immune response is associated with a rapid up-regulation of the expression of glucose transporters on the T cell membrane and a corresponding increase in glucose uptake. Antigen receptor-activated T cells also change from metabolizing glucose primarily through oxidative phosphorylation to also using the glycolytic pathway (3, 11). T cell immune responses are initiated in response to TCR antigen receptor triggering by cognate peptide/major histocompatibility complexes on the surface of antigen-presenting cells. Moreover, the T cell response to antigen is modulated by multiple adhesion molecules and co-stimulatory molecules and by multiple pro-inflammatory cytokines. These antigen receptor and co-stimulatory molecules coordinate the signaling pathways that control the changes in glucose metabolism that underpin T cell immune responses.

The up-regulation of glucose transporter expression and the glycolytic program of T cells is initiated by antigen and co-stimulatory molecules via the transcription factor c-myc (12). However, the expression of glucose transporters and glycolytic enzymes by antigen-activated T cells can also be sustained by inflammatory cytokines such as interleukin 2 (13–15). One key signaling pathway that sustains glucose metabolism is mediated by the serine kinase mammalian target of rapamycin complex 1 (mTORc1) via regulation of hypoxia-inducible factor-1 (HIF-1) complexes (12, 14, 16). The mTORc1/HIF pathway sustains glucose metabolism in IL-2-activated $CD8^+$ T cells by controlling expression of the glucose transporter Glut1 and by regulating expression of hexokinase 2, a key enzyme which phosphorylates glucose to produce glucose-6-phosphate, an essential intermediate in most pathways for glucose metabolism. The mTORc1/HIF pathway also controls expression of rate-limiting glycolytic enzymes in effector T cells such as phosphofructokinase 1, lactate dehydrogenase, and pyruvate kinase M2 (14).

The switch to glycolysis that accompanies T cell activation makes effector T cells dependent on relatively high levels of glucose transporter expression and exogenous glucose in order to sustain their transcriptional program (17, 18). There is not a

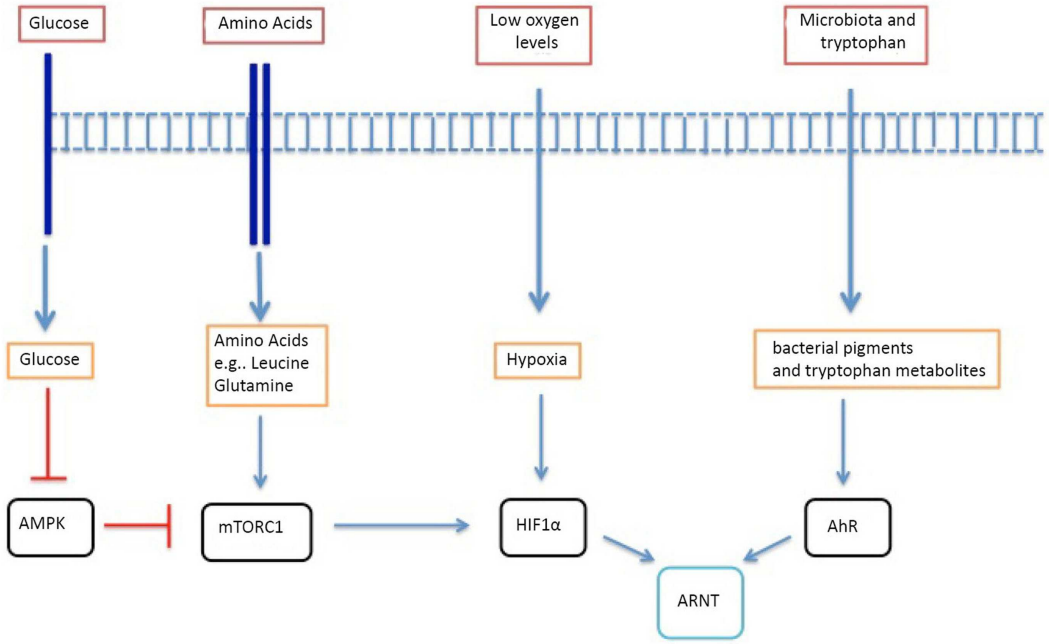

FIGURE 1 | A summary of the key metabolic and environmental sensors that influence T lymphocyte biology. mTORc1 is inhibited by AMPK, which is a key glucose sensor. mTORc1 is also influenced by intracellular amino acid levels such as leucine and glutamine. Oxygen tensions are sensed by HIF-1α, which remains under the control of mTORc1. Bacterial virulence factors and metabolites from dietary tryptophan are sensed by the AhR. AhR and HIF-1α have the same dimerizing partner – HIF-1β.

full understanding of how glucose metabolism controls T cell differentiation. However, one key glucose sensor in T cells is the adenosine monophosphate-activated protein kinase (AMPKα1) (19). This kinase is phosphorylated and activated by liver kinase B1 (LKB1) in response to the increases in cellular AMP:ATP ratios that occur rapidly when metabolically active T cells are glucose deprived (8, 20). The expression of AMPKα1 is not essential for effector T cell proliferation or differentiation but it is necessary for the survival of activated T cells *in vivo* following withdrawal of immune stimulation (8). AMPKα1 null $CD8^+$ T cells also show a striking defect in their ability to generate memory cells following pathogen infection. The importance of AMPK for T cells reflects its ability to enforce quiescence to limit energy demands under conditions of energy stress. Hence, a key role for AMPKα1 is to restrain the activity of the mTORc1 (8, 21, 22).

Adenosine monophosphate-activated protein kinase can also stimulate autophagy (23) and in this respect, autophagy has been shown recently to be critical for the formation of CD8 T cell memory (24, 25). The loss of key molecules that control T cell autophagy thus pheno-copies the impact of AMPK deletion on the formation of memory T cells.

Why is it important that activated T cells switch on glycolysis? The glycolytic pathway is a very inefficient way to produce ATP from glucose and it would seem more logical to use oxidative phosphorylation as long as oxygen tensions are sufficient. One explanation is that glycolytic intermediates are used as precursors for nucleotide, amino acid, phospholipid, and triglyceride biosynthesis. It is also noteworthy that non-metabolic functions of glycolytic enzymes have been described (26). For example, it has been described that the glycolytic enzyme GAPDH controls effector T cell production of the cytokine interferon gamma by binding to AU-rich elements within the 3′ UTR of IFN-γ mRNA and hence controlling the translation of this mRNA (18). One other factor to consider is that the glycolytic products lactic acid and succinate can function as "signaling" molecules to control transcriptional responses in macrophages and could well have similar functions in T cells (27, 28).

OXYGEN SENSORS AND T CELLS

One important environmental factor for T cells is the local oxygen (O_2) tension. The term hypoxia is used to refer to oxygen tensions below the physiological norm and it is now recognized that naïve T lymphocytes recirculate through tissues with quite wide ranges of oxygen saturation. Oxygen tension is thus relatively low in secondary lymphoid tissues such as lymph nodes compared with the arterial bloodstream (5 versus 13%) (29). Other tissues that have comparative hypoxia in healthy hosts include the intestine and skin (30, 31). It is also clear that effector T cells have to function under relatively hypoxic conditions, e.g., at sites of tissue inflammation and within tumor microenvironments where cellular growth rates supersede rates of angiogenesis and oxygen supply.

The main oxygen sensor in T cells is the transcription factor hypoxia-inducible factor alpha (HIF-1α). At atmospheric oxygen tension (21%), HIF-1α is rapidly degraded. This rapid degradation occurs because proline residues of HIF-1α become hydroxylated by prolyl hydroxylases after which HIF-1α is ubiquitinated by the von-Hippel–Lindau (Vhl) E3 ligase complex (32, 33) with the resultant targeting of HIF-1α for degradation. The hydroxylation of HIF-1α

requires oxygen as a substrate (34) such that HIF-1α degradation is inhibited when oxygen tension are low. Stabilized, HIF-1α translocates to the nucleus where it dimerizes with HIF-1β (also named the aryl hydrocarbon nuclear translocator). The HIF-1α/HIF-1β heterodimer then binds to hypoxia response elements (HREs) in the promoters of specific genes (29).

In both $CD4^+$ and $CD8^+$ T cells, HIF complexes accumulate in effector T cells even under normoxic conditions if these cells have high levels of mTORc1 activity (14, 16). This probably reflects that mTORc1 controls the translation of HIF-1α mRNA. Nevertheless, expression of HIF-1α in effector T cells can also be rapidly enhanced further by exposure to hypoxia (14). The HIF-1 pathway is required to sustain expression of multiple genes encoding proteins that control glycolysis and pyruvate metabolism in effector T cells. The expression of the glucose transporter GLUT1 is HIF controlled in T cells but the ability of HIF-1 to sustain glucose metabolism extends beyond a simple model of HIF-1 regulation of glucose uptake. HIF-1 null effector cytotoxic T lymphocytes (CTL) thus cannot sustain expression of multiple rate-limiting glycolytic enzymes; hexokinase 2, pyruvate kinase 2, phosphofructose kinase, and lactate dehydrogenase. Strikingly, HIF-1 regulates a quite diverse transcriptional program in CTL and in particular controls expression of cytolytic effector molecules such as perforin and granzymes. Indeed, when CTL are switched from normoxic (21%) to hypoxic (1%) oxygen, they substantially increase expression of HIF-1α and perforin (14). These results explain observations that CTL cultured under hypoxic conditions display increased cytotoxic function (**Figure 2**) (35).

It is also of note that HIF-1-regulated genes in CTL encode chemokine receptors and adhesion molecules. For example, HIF activity regulates the expression of the chemokine receptor CCR7 and the cell adhesion molecule CD62L (L-selectin). These molecules are expressed at high levels in naïve and memory T cells and are essential for lymphocyte transmigration from the blood into secondary lymphoid tissue. In contrast, effector CTL downregulate CD62L and CCR7 expression as part of the program that redirects effector T cell trafficking away from lymphoid tissue toward sites of inflammation (14). In the absence of HIF transcriptional complexes, effector CTL retain expression of CD62L and CCR7 and they also retain the migratory properties of naïve/memory T cells and preferentially home to secondary lymphoid tissues (14). There is therefore a dominant requirement for HIF-1 for the normal programing of effector CD8 T cell trafficking.

HIF transcriptional complexes, the metabolic sensor of cellular oxygen levels, thus act positively to control expression of glucose transporters and cytolytic effector molecules but have a role to repress expression of critical chemokine and adhesion receptors that regulate T cell trafficking. There are thus fundamental mechanisms mediated by HIF that link oxygen sensing and transcriptional control of CD8 T cell differentiation. There is moreover evidence that HIF signaling is an important regulator of effector CD8 T cell function. Hence, T cell-specific deletion of Vhl, which targets HIF for degradation causes increased pathology in response to chronic viral infection (36) reflecting that HIF signaling seems to limit the terminal differentiation of CD8 T cells.

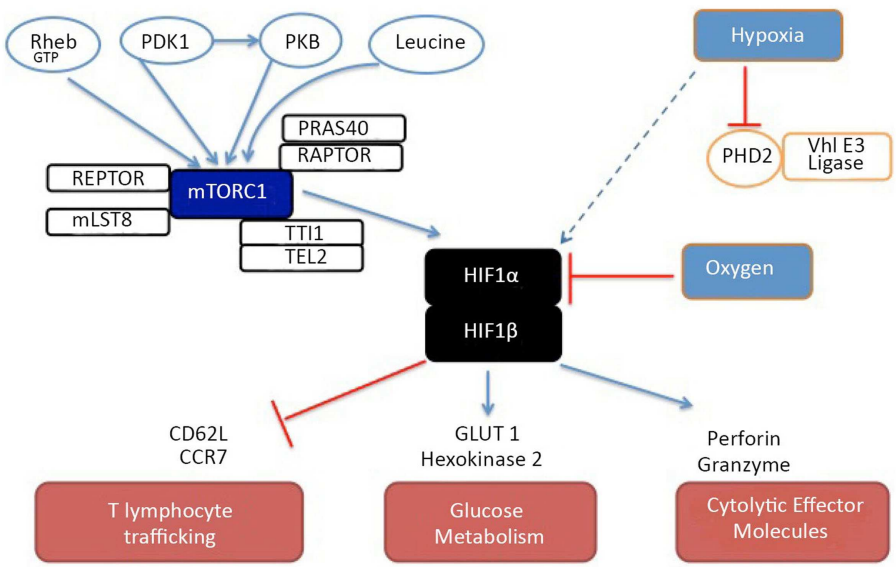

FIGURE 2 | A model of the regulation and function of HIF complexes in cytotoxic T cells. Under normoxic conditions, HIF-1α is rapidly degraded. This rapid degradation occurs because proline residues of HIF-1α become hydroxylated by prolyl hydroxylases after which HIF-1α is ubiquitinated by the von-Hippel–Lindau E3 ligase complex. In hypoxia, the hydroxylation of HIF-1α does not occur and the transcription factor is no longer targeted for degradation and HIF-1α protein can thus accumulate within the cell. In effector CD4 and CD8 T cells, high levels of mTORC1 activity promote HIF-1α translation allowing moderate levels of HIF complexes to accumulate even under normoxic conditions. The different regulatory signals that control mTORc1 activity in T cells are also shown as are some of the gene targets whose expression is either promoted (blue arrows) or repressed (red lines) by HIF-1 complexes.

There are parallel data that oxygen sensing acts via HIF to control the differentiation of effector CD4 cells (16). The oxygen tension in the T cell environment will thus have an impact of the T cell differentiation program.

REGULATION OF AMINO ACID TRANSPORT IN T LYMPHOCYTES

T cell activation is known to increase rates of amino acid uptake by increasing expression of key amino acid transporters. For example, immune-activated T cells up-regulate expression of glutamine transporters; members of the sodium-dependent neutral amino acid transporter (SNAT) family (5, 7). In the context of glutamine uptake, T cells have an absolute requirement for a large external supply of glutamine and even modest reductions of external glutamine concentrations will negatively impact on T cell proliferation and differentiation (7). Moreover, triggering of the TCR induces expression of the glutamine transporter ASCT2 in T cells and the deletion of ASCT2 suppressed the development of pro-inflammatory CD4 helper 1 (Th1) and Th17 cells *in vivo* in mouse models of autoimmunity highlighting the integration of T cell responses to antigen with changes in T cell glutamine metabolism (7).

Exposure to pathogens and triggering of the TCR in $CD8^+$ T cells also induces a striking increase in expression of the System L amino acid transporter complex that comprises a heterodimer of CD98 and the large neutral amino acid transporter (LAT1), encoded by the SLC7A5 gene (5). The regulated expression of System L amino acid transporters is particularly important for T cells as these are responsible for cellular uptake of branched chain and aromatic amino acids such as leucine, isoleucine, tryptophan, and phenylalanine (37). These amino acids are all used for *de novo* protein synthesis. System L transport activity in T cells requires sustained exposure to antigen/pathogen or inflammatory cytokines such as interleukin 2 (IL-2) and is an example of how the T cell response to immune stimuli controls the ability of T cells to respond their environment (5).

The regulated expression of acid transporters is particularly important for T cells as these mediate the cellular uptake of the amino acids required for *de novo* protein synthesis. T cell activation is thus associated with large increases in protein synthesis to support the replication of cellular proteins during cell division and to support the synthesis of the secreted cytokines, chemokines, and effector molecules. For example, there is a requirement for sustained leucine uptake for expression of the metabolic regulator c-myc. Slc7a5-null T cells can thus respond to antigen and cytokines to increase expression of mRNA encoding c-Myc but they do not express c-Myc protein. Accordingly, Slc7a5-null T cells are unable to up-regulate the Myc-controlled "metabolic machinery" required for T cell differentiation (5). For example, Slc7a5-null T cells are unable to increase glucose and glutamine uptake in response to TCR antigen receptor engagement.

One important factor in the context of glutamine metabolism in T cells is that the majority of glutamine taken up by T cells is not used for *de novo* protein synthesis. Instead, it is diverted into metabolic intermediates such as pyruvate and particularly lactate with a small amount being oxidized to carbon dioxide through the citric acid cycle. The conversion of glutamine to lactate occurs via a metabolic process known as glutaminolysis whereby glutamine is metabolized to glutamate which is then converted into α-ketoglutarate. Glutamine metabolized in this way is not only a metabolic fuel but provides substrates for T cells to grow and proliferate (12). For example, α-ketoglutarate can enter an isocitrate dehydrogenase-1 (IDH1)-dependent pathway to synthesize acetyl coenzyme A (AcCoA): a key biosynthetic precursor for fatty-acid synthesis and protein acetylation. Here, it is relevant that AcCoA can be produced from glucose-derived pyruvate but in any cell that has switched to aerobic glycolysis, there is the possibility of a stoichiometric conversion of glucose to lactate which will direct glucose-derived carbon away from the tricarboxylic acid cycle and fatty-acid synthesis. Under these conditions, cells may become completely dependent on IDH1-mediated reductive carboxylation of glutamine-derived α-ketoglutarate for *de novo* lipogenesis. Note, α-ketoglutarate also provides the link between glutamine and the Krebs cycle and here it has been noted that T cell activation is associated with increased expression of key enzymes such as involved in glutamine/α-ketoglutarate metabolism notably glutamate dehydrogenase (GDH), glutamic-oxaloacetic transaminase (GOT), and glutamic-pyruvic transaminase (GPT) (38).

It is also noteworthy that mutations in (IDH1) and IDH2 have been found in acute myeloid leukemias (AMLs). These mutant IDH enzymes have a gain of function activity to catalyze the NADPH-dependent reduction of α-ketoglutarate to 2-hydroxyglutarate (2HG). This latter metabolite can act as a competitive inhibitor of histone lysine demethylases and hence control histone demethylation and chromatin structure (39). These observations reveal a clear link between metabolic processes and the cellular epigenome and afford another insight as to how metabolic signaling can impact on cellular transcriptional programs.

MAMMALIAN TARGET OF RAPAMYCIN: AN AMINO ACID SENSOR IN T CELLS

One of the main reasons that it is critical that T cells tightly regulate expression of System L amino acid transporters and glutamine transporters is because the intracellular supply of leucine and glutamine regulates the activity of the serine/threonine kinase complex mTORc1 (2, 40–42). The leucine-sensing pathway that activates mTORc1 activity is initiated within lysosomes and involves amino acid-dependent activation of the guanine nucleotide exchange activity of the Ragulator complex. This results in the accumulation of active GTP-bound RagA GTPases, which then recruits mTORc1 to the lysosomal surface where it interacts with the small GTPase Rheb, a potent stimulator of mTORc1 kinase activity in T cells (43, 44). The full details of how T cells sense intracellular leucine are not known but it importance and the importance of mTORc1 for effector T cell differentiation has been well documented (2, 5, 45). The molecular basis for the immunosuppressive actions of mTORC1 inhibitors such as rapamycin is multifaceted. In CD4 T cells, rapamycin inhibition of mTORC1 prevents the differentiation of Th1 and Th17 but not Th2 cells. Rapamycin also causes retention or re-expression of FoxP3 in antigen receptor-activated CD4 T cells and promotes the production of "regulatory" T lymphocytes (2, 45). Inhibition of mTORC1 with rapamycin can also shape CD8 T cell responses to antigen: rapamycin treatment thus

prevents effector T cell generation and promotes memory T cell formation (46).

The molecular basis for the importance of mTORc1 for T cell function is not fully understood and is not the focus of the present review. However, as discussed above, one key function for mTORc1 is to control expression of HIF-1 transcription factor complexes which then regulate expression of a diverse array of genes including glucose transporters, glycolytic enzymes, adhesion molecules, and cytolytic effector molecules (14, 16). mTORc1-controlled signaling pathways can also control expression of the sterol regulatory element-binding proteins (SREBP1 and SREBP2); transcription factors that are required for the expression of lipogenic enzymes (47). mTOR has thus been linked to the control of lipid biosynthesis in many cells. The relevance of the SREBP pathway in T lymphocyte biology was suggested when it was demonstrated that phospholipid-dependent kinase 1 (PDK1), which controls mTORc1 activity in CD8 T cells (14), controls the transcription of SREBP gene targets such as hydroxysteroid (17β) dehydrogenase 7, fatty-acid desaturase 2, farnesyl diphosphate synthetase, and stearoyl-coenzyme A desaturase 1 (13). Moreover, SREBP null CD8 T cells cannot metabolically reprogram to glycolysis and show attenuated clonal expansion in response to a pathogen challenge (48). In this context, there is an increasing awareness that the regulation of *de novo* fatty-acid synthesis and fatty-acid catabolism is important for peripheral T cell differentiation (49–51).

MICROBIOME SENSORS AND T CELLS

All mammals are colonized with diverse commensal microbes especially at barrier sites such as the skin and the gastrointestinal tract and it is now recognized that these resident bacteria have a key role in controlling immune cell homeostasis and can modulate the function of T cells in adaptive immune responses (52, 53). Importantly, commensal bacteria constantly trigger responses in gut epithelial cells via host microbial pattern recognition receptors particularly Toll-like receptors (TLRs). These signals are essential under normal non-pathogenic steady-state conditions to maintain intestinal epithelial homeostasis (54). Commensal microbes are also essential for controlling the function of lymphocytes at barrier sites. For example, germ-free mice have smaller Peyer's patches and fewer T and B cells in the intestine than mice with a normal microbial flora. It has also been shown that the differentiation of effector T cell populations can be influenced by the gut microflora. For example, segmental filamentous bacteria (SFB) that adhere tightly to the epithelial layer of the intestine have been shown to drive the differentiation of mucosal Th17 cells (55–58). As well, the maintenance of regulatory T lymphocytes in the gut can be modulated by intestinal bacterial colonization (59).

The molecular mechanisms whereby the bacterial environment controls T cells are complex and a full analysis is beyond the scope of the present review. One element is that bacterial recognition by pattern recognition receptors expressed by epithelial cells, macrophages, and dendritic cells will govern the production of cytokines and chemokines that can direct T cell fate (60, 61). Hence, the nature of the pathogen insult/challenge to the innate immune system, and the resultant cytokine milieu plays a key role in regulating peripheral T cell differentiation. One more direct pathway that interprets the microbial environment for T cells is mediated by the aryl hydrocarbon receptor (AhR), a transcription factor that has been extensively studies in the context of cellular responses to environmental toxins. Ligands for the AhR include dioxins and in the context of toxin binding the AhR translocates to the nucleus to form a complex with the AhR nuclear translocator (ARNT) or HIF-1β. This complex then regulates expression of members of the cytochrome P450 family such as CYP1A1, which function as xenobiotic metabolizing monooxygenases (10). In the context of the influence environmental cues have of lymphocyte population, it is of interest that both HIF-1α and AhR have the same dimerizing partner HIF-1β/ARNT. This suggests a potential for cross talk between the hypoxia-induced factors and the AhR pathways.

The AhR has an important physiological role in T cell immune responses and can regulate the differentiation of effector CD4 T cells that make the pro-inflammatory cytokines IL-17 and IL-22 (62). The AhR also controls the development of innate lymphoid cells (ILCs) at barrier sites such as the intestinal mucosa and the skin (63). The direct gene targets for the AhR that explain how it controls T cell differentiation are not known but could be the cytokine gene promoters themselves.

The AhR was first studied in the context of environmental toxins such as dioxins [e.g., d2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) ligands]. However, it is the identity of physiologically relevant AhR ligands that has captivated the immunological community. One key discovery was that exposure of tryptophan to ultraviolet light from solar irradiation (in particular UVB) results in the production of a potent AhR ligand 6-formylindolo(3,2-b)carbazole (FICZ) (10). Moreover, AhR null mice have a 90% reduction of TCRγδ$^+$ skin T cells. (64). In addition, activation of the AhR dampens the severity of immune-mediated skin inflammation (65).

It has been proposed that AhR activity in gut lymphocytes can be regulated by the metabolism of nutritional components notably tryptophan catabolites produced by diets rich in cruciferous vegetables (66). One product of tryptophan metabolism that functions as an AhR ligand is kynurenine (67, 68). In this respect, high levels of kynurenine production by human tumors have been proposed to suppress antitumor immune responses and hence promote tumor-cell survival (67). The generation of kynurenine from tryptophan metabolism occurs via the enzymes indoleamine 2,3-dioxygenase (IDO) and tryptophan 2,3-dioxygenase (TDO).

The production of AhR ligands from tryptophan metabolites could explain how the diet can shape gut immune responses. The AhR is thus important for the development and maintenance of multiple subpopulations of gut lymphocytes. (69). However, one consideration is that diet alterations might change the bacterial milieu and indirectly regulate gut lymphocytes by controlling the diversity of gut commensal bacteria. In this respect, there is an increasing recognition that the bacterial microbiome can produce ligands for the AhR notably tryptophan metabolites. The AhR can thus act as a link between the host immune system and the commensal microbiota. There is also recent evidence that AhR is able to sense pathogenic microbes. Bacterial-pigmented virulence factors, namely phenazines from *Pseudomonas aeruginosa* and the naphthoquinone phthiocol from *Mycobacterium tuberculosis*, are thus strong AhR ligands. Importantly, there is

evidence that AhR null mice are more prone to infections with these organisms (70).

CONCLUSION

T cell responses to pathogens can be shaped by the localized microenvironment. T cell differentiation can be regulated by the bacterial environment because many of the cytokines and chemokines produced when pattern recognition receptors in cells of the innate immune system are triggered by bacterial products and subsequently control the adaptive immune response by acting on T cells. What is now emerging is that the bacterial environment can more directly regulate T cell responses. For example, T cells can be directly regulated by bacterial products that activate the AhR transcriptional pathway.

One other advance in our understanding of T cell biology is how the oxygen and nutrient environment can shape T cell differentiation. Moreover, that T cells are not just passive in the way they interact with their environment. T cells can thus control how they respond to the environment by restricting expression of high levels of nutrient receptors and environmental-sensing transcription factors to immune-activated T cells. The value of these insights is that they inform novel strategies to therapeutically manipulate T cell immune responses. For example, metformin, a drug that inhibits the respiratory chain complex I can promote the development of memory CD8 T cell-mediated immune responses arguing that it might be useful tool in vaccine development (71).

Tactics that promote signaling via the oxygen sensor HIF-1α have also been shown to promote the production of effector CD8 T cells, which could be important for strategies aimed at the clearance of viruses and tumors. In a similar context, strategies that activate the AhR have been shown to reduce inflammation in autoimmune-mediated skin conditions. Metabolic diseases such as obesity and diabetes could also be managed by modulation of the immune system. Recent discoveries have identified that innate lymphoid cell 2 subset (ILC2s) dysregulation is a conserved feature of obesity and manipulation of the IL-33/ILC2 axis may lead to therapies for this disease (72). The field of immunotherapy is entering a new era where targeting of pathways that influence the way T cells sense their environment may yield immunemodulatory drugs that are as valuable as those that currently target T cell-specific antigen and cytokine receptor signaling molecules.

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mTOR links environmental signals to T cell fate decisions

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T cell fate decisions play an integral role in maintaining the health of organisms under homeostatic and inflammatory conditions. The localized microenvironment in which developing and mature T cells reside provides signals that serve essential functions in shaping these fate decisions. These signals are derived from the immune compartment, including antigens, co-stimulation, and cytokines, and other factors, including growth factors and nutrients. The mechanistic target of rapamycin (mTOR), a vital sensor of signals within the immune microenvironment, is a central regulator of T cell biology. In this review, we discuss how various environmental cues tune mTOR activity in T cells, and summarize how mTOR integrates these signals to influence multiple aspects of T cell biology.

Keywords: mTOR, T cells, iNKT cell, T_{reg} cells

INTRODUCTION

T lymphocytes are comprised of heterogeneous populations that include conventional αβ T cells, γδ T cells, invariant natural killer T (iNKT) cells, and Foxp3$^+$ regulatory T (T_{reg}) cells. These functionally and phenotypically distinct T cell populations are involved in immune homeostasis and tolerance, pathogen clearance, and elimination of cancerous cells. T cell fate decisions are shaped by environmental signals received from nutrients, growth factors, cytokines, and cell–cell interactions. The serine/threonine kinase, mechanistic target of rapamycin (mTOR; formerly known as the mammalian target of rapamycin), integrates these environmental cues. The mTOR kinase exists in two, multi-protein complexes: mTOR complex 1 (mTORC1) where mTOR associates with Raptor, or mTOR complex 2 (mTORC2) where Rictor and mSin1 bind mTOR (1, 2). mTORC1 activity is sensitive to, while mTORC2 activity is largely insensitive to, rapamycin treatment. Additionally, the upstream activating stimuli and downstream effector functions differ between these complexes (1, 2).

While the signaling pathways inducing mTORC2 activation in T cells are poorly understood, in other cell lineages, mTORC2 associated with ribosomes is strongly activated, while ER stress or GSK3-β-mediated phosphorylation of Rictor inhibits its activation (3, 4). Upstream positive regulators of mTORC1 activation include the PI3K–PDK1–Akt pathway, the RasGRP–Ras–MAPKK (also known as MEK)-ERK1/2 kinase cascade, and the small GTPase, RHEB. By contrast, the phosphatase, PTEN, TSC1/TSC2, and the LKB1–AMPK pathway antagonize mTORC1 function (1, 2).

When activated, mTORC1 signaling promotes S6K function and suppresses 4E-BP1 activation, while mTORC2 regulates Akt, SGK1, and PKC catalytic activity (1, 2, 5–8). mTOR signaling also activates transcription factors, such as c-MYC, hypoxia inducible factor 1-α (HIF1-α), and sterol regulatory element-binding proteins (SREBPs) (1, 2). Ultimately, the activation of mTOR-induced pathways impacts gene expression, protein translation, cell metabolism, growth, proliferation, survival, or migration in multiple cell lineages, including T lymphocytes (1, 2). Because of these critical biological effects, dysfunctional mTOR signaling is also linked to autoimmunity, obesity, and cancer, among other conditions (2, 9, 10).

Here, we review the multifactorial roles of mTOR in T cell biology. We first discuss how different environmental stimuli activate mTOR within T cells. Second, we describe the role of mTOR in thymocyte development. We then reveal how mTOR function is coupled to peripheral T cell quiescence, functional activation, and differentiation. The ability of mTOR to dampen the immune response by modulating T_{reg} cell function is also discussed. We then review the known functions mTOR serves in regulating T cell trafficking under homeostasis and upon infection. Finally, we highlight how future studies will further advance our understanding of mTOR functions in T cells, and how these findings may be applied therapeutically.

MULTIPLE SIGNALS WITHIN THE IMMUNE MICROENVIRONMENT TUNE mTOR ACTIVITY IN T CELLS

Specialized signals derived from immune microenvironments shape T cell biology. To develop into mature T cells or gain effector functions, T cells require stimulation by immune receptors, including the TCR and co-stimulatory receptors. Soluble factors, such as cytokines, adipokines, growth factors, and nutrients, also affect T cell development and functional activation (1). mTOR integrates these immunological and environmental cues to ultimately shape T cell development, activation, and differentiation into effector or long-lived, antigen-experienced memory T cells. Below, we discuss how various factors within the immune microenvironment tune mTOR activity, and a select summary of these pathways is shown in **Figure 1**.

TCR AND CO-STIMULATORY RECEPTORS

When occurring in the presence of co-stimulation, TCR recognition of self and non-self peptides expressed in the context of MHC molecules is critical for T cell development and functional activation, respectively (11–15). TCR and co-stimulatory receptor triggering activate mTOR in multiple thymocyte populations, peripheral CD4$^+$ and CD8$^+$ T cells, and Foxp3$^+$ T_{reg} cells.

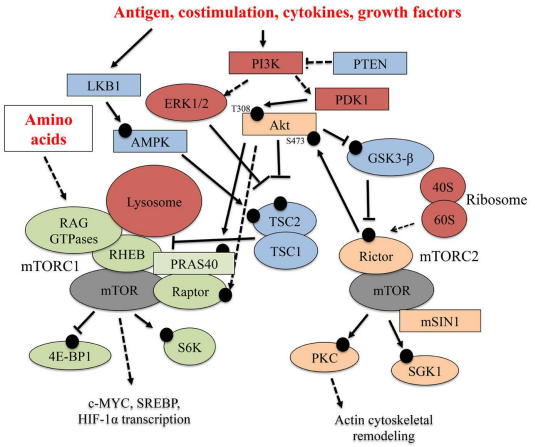

FIGURE 1 | Select upstream regulators and downstream effectors of mTOR signaling. Multiple signaling pathways emanating from the TCR, co-stimulatory receptors, cytokines, and nutrients (amino acids) tune mTOR activation in T cells. In this figure, black circles represent phosphorylation events. Solid arrows indicate a direct, activating phosphorylation event mediated by an upstream kinase, while dashed arrows indicate an indirect, positive regulatory role for a protein in a particular pathway. Inhibitory phosphorylation events or control of pathway activation are indicated by solid or dashed flat-ended arrows, respectively.

Many studies have aimed to elucidate the mechanisms underlying this activation. PI3K catalyzes the conversion of membrane-bound phosphatidylinositol (PtdIns)-(4,5)-bisphosphate (PIP_2) into PtdIns-(3,4,5)-triphosphate (PIP_3), which acts as a second messenger to recruit the enzymes, PDK1 and Akt, to the plasma membrane (13). As we discuss in greater detail below, the PI3K–PDK1–Akt signaling axis promotes mTORC1 activation by inactivating the TSC1/TSC2 complex, thereby driving RHEB activation (2). However, downstream of the TCR, RHEB is only required for early (e.g., during the first 4 h of stimulation) mTORC1 activation (16), suggesting further mechanisms by which PI3K–Akt regulates mTORC1 activation. In this regard, PRAS40 is a steric inhibitor of mTOR, and its direct phosphorylation by Akt releases its suppressive activity and promotes mTOR activation (17). Akt also indirectly promotes mTOR phosphorylation by inducing IκB kinase α (IKKα) activity, resulting in the formation of stable Raptor–mTOR interactions that support mTORC1 function (18). The requirement for Akt in regulating mTOR activation may differ between different T cell populations, as TCR-induced mTORC1 activity is controlled by a PI3K–PDK1-dependent, Akt-independent pathway in effector $CD8^+$ T cells (19). This Akt-independent pathway is linked to IL-2 induced metabolic reprograming and T cell proliferation (20). PI3K–Akt signaling is antagonized by PTEN, and loss of PTEN enhances mTOR activation (1, 2). Thus, the PI3K signaling axis is a critical regulator of mTORC1 activation at multiple levels.

In addition to PI3K, the modification of membrane-associated lipids is also controlled by PLC-γ1. Early after TCR stimulation, PLC-γ1 is activated, resulting in the cleavage of PIP_2 into inositol triphosphate (IP_3) and diacylglycerol (DAG). DAG supports the functional activation of the RasGRP–Ras–MAPKK–ERK1/2 pathway (13), and may cooperate with mTORC2 to induce PKC-θ activity (5). The Ras–MAPKK–ERK1/2 pathway promotes mTORC1 activation via the ERK1/2-dependent phosphorylation of TSC2 (21). T cells that lack DAG kinase (DGK)-α and DGK-ζ, which terminate DAG signaling, have elevated mTORC1 and mTORC2 activation (22). However, whether the DAG–RasGRP–Ras–MAPKK–ERK1/2 pathway acts independently or in concert with PI3K signaling is unknown, as the catalytic function of PI3K positively regulates TCR-induced ERK1/2 activation in mouse and human T cells (23, 24). Inducible Tec kinase (Itk), which directly phosphorylates and activates PLC-γ1, also promotes TCR-induced mTOR activation by inducing microRNAs that suppress PTEN expression (25). These studies indicate that many signaling pathways regulate mTOR activity downstream of the TCR.

Although TCR stimulation is necessary for effective T cell development and activation, co-stimulatory receptors must also be ligated to fully promote these processes and overcome a state of TCR-induced hypo-responsiveness called anergy (13). The classical co-stimulatory receptor for naïve T cells is CD28, which binds CD80–CD86 on antigen presenting cells (APC). However, other co-stimulatory receptors are expressed on activated T cells and T_{reg} cells, including OX40 and ICOS (26). OX40 has been demonstrated to augment TCR-induced PI3K activation to potentiate and sustain mTORC1 activity (27), further demonstrating the critical importance of the PI3K pathway in tuning mTOR activation.

Non-enzymatic proteins also regulate mTOR activation in response to antigen and co-stimulation. The CARD-containing membrane-associated protein 1 (CARMA1)-mucosa-associated lymphoid tissue lymphoma translocation protein 1 (MALT1) scaffolding complex is a recently identified, positive regulator of mTORC1 activation (28, 29). Because IKKα is known to associate with these proteins (14), this scaffolding complex may regulate the IKKα-dependent phosphorylation of mTORC1 in T cells. Additionally, the Hsp90 chaperone protein prevents Raptor protein degradation, thus promoting mTORC1 activation downstream of the TCR (30). However, the detailed mechanism by which Hsp90 prevents Raptor degradation remains unexplored.

CYTOKINES

The cytokine milieu is another crucial environmental component regulating T cell fate decisions. Within the thymus and in the periphery, IL-7 signaling via IL-7R drives T cell development and homeostasis, respectively (31). In a STAT5-dependent manner, IL-7 promotes low, transient mTORC1 activation that is critical to support IL-7 function in conventional T cells (32, 33). IL-12 activates mTOR via a STAT4-dependent mechanism in activated $CD8^+$ T cells (34), while IL-4 and IL-1 promote mTOR activation in T_H2 and T_H17 cells, respectively, to induce cell cycling (35, 36). The cytokine IL-15 regulates memory T cell formation (31, 37); however, although it activates mTOR via the PI3K pathway, IL-15-induced mTOR activation driving naïve, $CD8^+$ homeostatic proliferation is not necessary for memory T cell formation (38). Finally, IL-2 is a crucial cytokine that induces clonal expansion in activated T cells and supports T_{reg} cell development and function (31). After cells express high levels of the high affinity IL-2 receptor (e.g., CD25 coupled with CD127), IL-2 signaling strongly activates transcriptional and metabolic reprograming via the Jak3–STAT5 and PI3K–Akt–mTORC1 pathways (1, 31). Itk is

also required for efficient mTOR activation following IL-2 stimulation via mechanisms that are not fully elucidated (25). Like co-stimulatory receptor signaling in conventional T cells, IL-2 signaling also synergizes with TCR-dependent signals to enhance mTOR activation in T_{reg} cells (1, 39).

AMINO ACIDS

As we will discuss throughout this review, amino acids also regulate T cell activation. Relatively little is known about how amino acids control mTOR activation in T cells, but RHEB is an essential regulator of amino acid-induced mTORC1 activation in other cell lineages (40, 41). Mechanistically, amino acids drive mTORC1 activation by recruiting the heterodimeric complex of GTP-bound RAG GEFs (RAGA, RAGB, RAGC, and RAGD) to the lysosomes via the Ragulator complex (40, 41). This process is antagonized by the GAP activity of TSC2, which, when associated with lysosomes in the absence of PI3K–Akt signaling, inactivates RHEB (42). Indeed, TSC1-deficient T cells have hyper-elevated mTORC1 signaling (43), but it should be noted that amino acids can activate mTORC1 in a TSC1-independent fashion in other cell lineages (44).

Precisely how amino acids regulate T cell responses remains uncertain. In the absence of TCR and CD28 stimulation, amino acids promote mTORC1 activation in effector $CD8^+$ T cells (45). Moreover, amino acids enhance TCR and CD28-induced mTORC1 activation (29), and IL-7 or TCR and IL-2 stimulation also increases amino acids transport to promote efficient $CD8^+$ T cell responses (45). However, TCR and CD28-induced mTORC1 activation is controlled by RHEB-dependent and RHEB-independent mechanisms (16). One potential explanation for these data is that amino acids localize mTORC1 to the lysosome to potentiate the early activation of mTORC1 via RHEB. After prolonged antigen exposure, however, other TCR and CD28-induced signaling pathways are sufficient to sustain mTOR activation independently of RHEB (16). Future work will continue to dissect the mechanisms by which amino acids activate mTORC1 in T cells and other cell linages, but they may regulate CARMA1–MALT1–Bcl10 complex composition and function (28, 29).

NOTCH

NOTCH signaling promotes thymocyte proliferation and survival, and aids in their differentiation into terminally differentiated T cells (15). We discuss the process of thymocyte development in greater detail in the next section. Ligation of NOTCH activates mTOR activation through PI3K–Akt (46). Interestingly, aberrant NOTCH signaling is observed in both human and murine T cell acute lymphoblastic leukemia (T-ALL), and NOTCH inhibition in T-ALL lines suppresses mTOR activation by inhibiting c-MYC expression (47). However, the precise mechanisms by which this occurs remain undefined.

LEPTIN AND SPHINGOSINE 1-PHOSPHATE (S1P)

Leptin is an adipocyte-derived cytokine, or adipokine, and serves multiple roles in T cells as discussed throughout this review. Recently, it was demonstrated that leptin receptor signaling contributes to the high levels of mTORC1 signaling that inhibits their IL-2-induced proliferation *in vitro* (39, 48). We describe how mTOR controls T_{reg} cell development, differentiation, and function in a later section. The lipid chemokine, S1P, signals via S1PR1 and drives mTORC1 activation in a PI3K–Akt-dependent manner (49–51). These studies indicate that multiple, immune-mediated signals regulate mTOR activation within T cell populations. Below, we discuss how the integration of these signals via mTOR regulates T cell development, functional activation, suppressive function, and migration.

ROLE OF mTOR SIGNALING IN THYMOCYTE DEVELOPMENT

OVERVIEW OF THYMOCYTE DEVELOPMENT

T cell development occurs within the thymus and results in the generation of mature, conventional αβ $CD8^+$ or $CD4^+$ T cells or non-conventional T cell populations, including $CD4^+$ $Foxp3^+$ thymic-derived T_{reg} (tT_{reg}) cells, γδ T cells, and iNKT cells. Thymocytes destined to become any T cell lineage begin as $CD4^-CD8^-$ double negative (DN) thymocytes, which can be further divided into substages: DN1, DN2a, DN2b, DN3a, DN3b, and DN4. NOTCH signals drive early proliferation and T cell lineage commitment by inducing expression of the pre-TCR (e.g., a rearranged TCRβ chain with a surrogate α chain) or the γδTCR in DN thymocytes. DN2 cells that upregulate the expression of the γδTCR in the presence of high levels of IL-7R signaling will become mature γδ T cells. By contrast, to develop into conventional αβ T cells, the DN3a cells must receive signals through the pre-TCR and NOTCH to undergo β-selection. DN cells next progress into the $CD4^+CD8^+$ double positive (DP) stage. Then, these cells receive positive and negative selection signals from the TCR to become $CD4^+$ or $CD8^+$ single positive (SP) cells. These SP will migrate to peripheral tissues as quiescent, mature $CD4^+$ or $CD8^+$ T cells. $Foxp3^+$ tT_{reg} cells differentiate from DP cells upon receiving intermediate affinity TCR signals in the presence of IL-2 and/or IL-15. The coordination of receptor-mediated signals and transcription factor networks driving T cell development are discussed in other reviews (14, 15).

iNKT cells are a specialized, non-conventional subset of αβ T cells, and are harmful or protective in a variety of diseases (12). In both humans and mice, the TCR repertoire is restricted to Vα18–Jα18 chain paired with a limited number of Vβ chains (12). This TCR recognizes lipid antigens expressed in the context of the non-classical MHC molecule, CD1d. iNKT cell development also occurs in the thymus, diverging from the conventional αβ T cells at the DP stage in response to strong, CD1d-presented TCR signals in combination with SLAM ligation (12). In mice, the development of these cells is tracked by the expression of CD24, CD44, and NK1.1: immature stage 0 ($CD24^+CD44^-NK1.1^-$), transitional stages 1 ($CD24^-CD44^-NK1.1^-$) and 2 ($CD24^-CD44^+NK1.1^-$), and mature stage 3 ($CD24^-CD44^+NK1.1^+$). The transcription factors PLZF, GATA3, T-bet, and ROR-γt are expressed at different levels in these stages, determining their IL-4-producing NKT-2, IFN-γ-producing NKT-1, and IL-17-producing NKT-17 cell fate commitments (12, 52). NKT-2, NKT-17, and NKT-1 cells are enriched in stages 1/2, stage 2, and stage 3, respectively (52).

mTOR CONTROLS CONVENTIONAL αβ T CELL DEVELOPMENT

To date, many studies have determined the impacts of mTOR inhibition at different stages of thymopoiesis. The conditional deletion of Raptor early during thymocyte development results

in less cell cycling and proliferation, more apoptosis, and severe thymic atrophy (53). By contrast, abrogation of mTORC1 function does not appear to affect later stages of thymocytes development, as no major developmental defects are observed when mTOR is deleted in the DP stage (54) or when Raptor is deleted in the DN3 or DP stage by Lck-Cre and CD4-Cre, respectively (16, 53). Thus, mTORC1 activation serves different functions throughout thymocyte development (**Figure 2**).

mTORC2 is also critical for thymocyte development, but it appears that the mechanisms by which mTORC2 supports thymocyte development differ from mTORC1 (**Figure 2**). Three different genetic models (e.g., whole animal, hematopoietic-specific deletion, and T cell precursor-specific deletion) have shown loss of Rictor at different stages compromises thymocyte development and leads to thymic atrophy (53, 55, 56). Mechanistically, mTORC2 activity is connected to the stability, *de novo* synthesis, and/or post-transcriptional modifications of proteins involved in thymic selection, including CD4, CD8, pre-TCR, TCR, NOTCH, and CD147, a receptor expressed on highly proliferative DN4 cells (56). Collectively, these studies reveal discrete functions of mTORC1 and mTORC2 in regulating thymocyte development.

Negative regulators of mTOR also influence T cell development. *Pten*$^{-/-}$ T cells undergo malignant transformation regulated, in part, by elevated Akt and mTOR activation (57, 58). PTEN deficiency does not affect conventional T cell development, although only CD4 SP thymocyte frequencies were reported (59). However, another study demonstrated that loss of PTEN leads to the accumulation of DN, DP, and CD4 SP thymocytes, and a reduction in negative selection at the DP stage (60). These data are in subtle contrast to the positive roles Akt and mTOR play in thymocyte development (16, 53–55, 61, 62). Work from our lab and others have shown that T cell-specific deletion of TSC1 does not inhibit thymocyte development (43, 63, 64). By contrast, *Lkb1*$^{-/-}$ thymocytes have a severe developmental block linked to defects in proliferation and survival (65, 66), but these effects appear to be independent of the known substrates of LKB1, AMPK1α or the related protein, MAP/microtubule affinity-regulating kinase 2 (MARK2) (65, 67, 68). Whether LKB1 controls thymocyte development via AMPK-independent pathways or AMPK family members are functionally redundant in thymocyte development is currently unresolved.

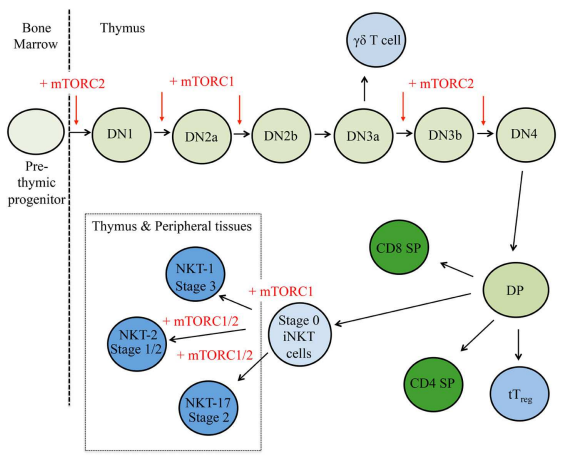

FIGURE 2 | mTOR is a critical regulator of thymocyte development. T cell progenitors first develop within the bone marrow and migrate to the thymus. Here, cells respond to multiple environmental stimuli and progress through CD4$^-$CD8$^-$ double negative (DN) stages 1–4 to the double positive (DP) stage. These DP thymocytes will then adopt different cellular fates in response to additional cues. Red arrows indicate where mTORC1 and/or mTORC2 control thymocyte fate decisions, where plus signs (+) represent positive regulation and minus signs (–) depict negative regulation.

mTOR SUPPORTS NON-CONVENTIONAL iNKT CELL AND T_{reg} CELL DEVELOPMENT

γδ T cell

Treating human peripheral blood mononuclear cells with rapamycin increases the TCR-driven expansion and effector functions of γδ T cell (69), while rapamycin treatment *in vivo* suppresses the functional activation of skin-resident, murine γδ T cells (70). However, the functional role mTOR signaling serves in γδ T cell development is currently unknown.

iNKT cells

mTORC1 and mTORC2 are critical regulators of iNKT cell development. *Rptor*$^{-/-}$ iNKT cells accumulate in stages 0 and 1, leading to a severe reduction of mature iNKT cells in the periphery (71, 72), whereas *Rictor*$^{-/-}$ iNKT cells are developmentally blocked at stage 2 (73, 74). The lineage commitment of iNKT cells is compromised by loss of Raptor, as the frequency of IFN-γ-producing, T-bet$^+$ NKT-1 cells is reduced (72). By contrast, Rictor deficiency does not diminish NKT-1 cell differentiation. Loss of Rictor, however, does suppress NKT-17 cell and/or NKT-2 cell development (73, 74). Mechanistically, mTORC1 regulates iNKT cell proliferation (72), whereas mTORC2 drives TCR-induced proliferation at stage 1 and protects from TCR-induced apoptosis (73, 74). These data indicate that mTORC1 and mTORC2 serve important, yet distinct, functions in iNKT cell development.

Elevated mTOR signaling also alters iNKT cell development. Compared to conventional T cells, iNKT cells express higher levels of *Tsc1* and *Tsc2* mRNA (75). Importantly, this high level of TSC1/TSC2 expression regulates the terminal maturation of iNKT cells, as *Tsc1*$^{-/-}$ thymocytes have severe limitations in developing past stage 2 and into functional NKT-1 cells (75). Recent work has also demonstrated that folliculin-interacting protein 1 (Fnip1) is required for iNKT cell progression beyond stage 2 (76). Mechanistically, *Fnip1*$^{-/-}$ iNKT cells are more sensitive to apoptosis, which may be attributed to excessive mTOR signaling and mitochondrial disruption (76). Finally, PTEN also regulates iNKT cell development and function. Suzuki and co-workers demonstrated that PTEN deficiency blocks progression from stage 2 to stage 3 and also abrogates TCR-induced IFN-γ production in these cells (77). Moreover, we have recently demonstrated that NKT-17 cell development is enhanced in the absence of PTEN, in part because mTORC2 signaling is elevated in these cells (74). These studies demonstrate a pivotal role for mTOR signaling in controlling iNKT cell development.

Foxp3$^+$ tT_{reg} cells

In addition to iNKT cells, Foxp3$^+$ tT_{reg} represent a non-conventional T cell population that develops within the thymus

(14). It has been reported that mTOR conditional knockout mice have normal frequencies of T_{reg} cells (54). Conditionally deleting PTEN within T cells does not dramatically alter T_{reg} cell development, although PTEN does suppress the IL-2-induced expansion of these cells (59). Moreover, TSC1 deficiency within the total T cell or T_{reg} cell compartments does not alter thymic or peripheral T_{reg} cell ratios (43, 78), but does impair their function as we discuss below. It is noteworthy that these studies did not distinguish between tT_{reg} and peripherally induced T_{reg} cells (pT_{reg}), which differentiate from naïve $CD4^+$ T cells following antigen stimulation in the presence of select cytokines. We discuss the pharmacological and genetic evidence linking mTOR signaling to pT_{reg} differentiation later in this review. Additional studies should explore the effects of LKB1–AMPK signaling on tT_{reg} cell development.

mTOR CONTROLS PERIPHERAL T CELL HOMEOSTASIS, ACTIVATION, AND DIFFERENTIATION

In the periphery, naïve T cells undergoing IL-7–IL-7R-driven homeostatic proliferation are maintained in a quiescent state (11). Upon receiving the appropriate antigen, co-stimulatory, cytokine, and nutrient signals, these T cells rapidly proliferate, generating multiple, antigen-specific T cell clones capable of inducing effective adaptive immune responses (13, 79, 80). These signals also induce the expression of transcription factors, including T-bet, GATA3, ROR-γt, Bcl-6, and Foxp3, which promote $CD4^+$ T helper (T_H)1, T_H2, T_H17, T follicular helper (T_{FH}), and pT_{reg} cell differentiation, respectively (79). Similarly, these signals drive $CD8^+$ T cell differentiation into short-lived effector T cells [SLECs; T-bet^{hi}Eomesodermin (EOMES)$^+$Blimp-1^{hi}KLRG1^+IL-7Rα^{lo}] or memory precursor cells (MPECs; T-$bet^{lo}$$EOMES^{lo}$Blimp-$1^{hi}$KLRG$1^{lo}$IL-7R$\alpha^{hi}$) (80, 81). The switch from naïve to activated to memory T cells is coordinated by an intricate network of epigenetic, transcriptional, and metabolic programs, many of which are directly influenced by mTOR activation (1, 82, 83). Below, we discuss how alterations in mTOR signaling affect mature T cell quiescence, functional activation, and differentiation. A summary is shown in **Figure 3**.

T CELL HOMEOSTASIS REQUIRES LOW LEVELS OF mTORC1 SIGNALING

Tonic TCR signaling induced by host-derived antigens in combination with IL-7R signaling maintains T cell homeostasis (11). Moreover, recent work has linked PI3K–Akt–mTOR signaling to the homeostatic proliferation of NKT-17 cells, which preferentially require IL-7 for their homeostasis (84). While mTOR, Raptor, or Rictor-deficient T cells have no alterations in steady-state peripheral T cell homeostasis (16, 54), low levels of mTOR signaling appear to maintain $CD4^+$ and $CD8^+$ T cell quiescence. In support of this idea, *Tsc1*$^{-/-}$ T cells have excessive mTORC1 signaling, which promotes aberrant cell cycling (43, 63, 64, 85). *Tsc1*$^{-/-}$ T cells have reduced homeostatic proliferation in response to IL-7 signaling and are hyper-responsive to TCR-induced apoptotic signals (43, 63, 64, 85). Bcl2 overexpression rescues this defect in apoptosis, but does not restore quiescence (43).

PTEN and LKB1 are also regulators of peripheral T cell homeostasis. Mature PTEN-deficient T cells are hyper-proliferative, resistant to apoptosis, and drive autoimmunity (86). Similar to *Tsc1*$^{-/-}$ T cells, peripheral *Lkb1*$^{-/-}$ T cells are hyper-activated and are more sensitive to TCR-induced apoptosis (87). Moreover, anti-CD3 and anti-CD28 antibody, but not IL-7, induced proliferation is impaired in the absence of LKB1 (66). Although TSC1 and LKB1 have similar defects, multiple metabolic pathways, including mitochondrial functions, are dysregulated in *Tsc1*$^{-/-}$ T cells (43, 63), while glycolysis is enhanced in the absence of LKB1 (87). Thus, TSC1 and LKB1 are both critical to maintain quiescence, but they control naïve T cell homeostasis by different mechanisms.

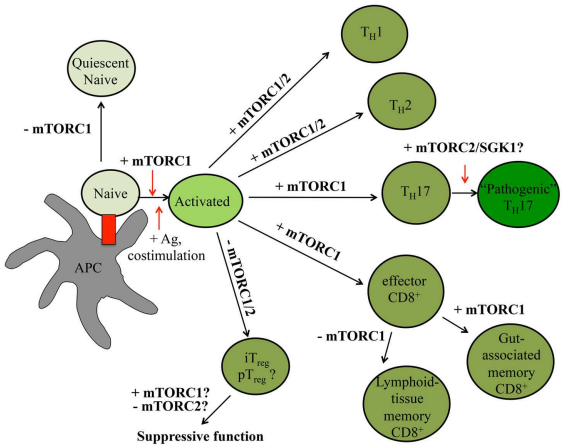

FIGURE 3 | mTOR signaling controls peripheral T cell fate decisions. In the peripheral tissues, T cell quiescence is controlled by low levels of mTORC1 signaling. Upon receiving antigen and co-stimulatory signals, T cells rapidly expand. In the presence of select cytokines, $CD4^+$ T cells further differentiate into different effector $CD4^+$ T cell lineages. $CD8^+$ T cells will become effector T cells before becoming memory T cells. The roles mTORC1 and mTORC2 serve in various T cell states are indicated within the figure, with positive roles shown with plus (+) signs and negative roles indicated by minus (−) signs. Question marks (?) indicate pathways requiring further investigation.

mTOR SIGNALING IS COUPLED TO T CELL CLONAL EXPANSION

It has been demonstrated that mTOR, RHEB, and Raptor-deficient T cells have defects in antigen-driven proliferation (16, 54). This effect is largely dependent upon mTORC1-mediated signaling driving cell cycle entry from quiescence, as loss of Raptor or rapamycin treatment in naïve, but not proliferating, T cells blocks clonal expansion and instead promotes T cell anergy (16, 88). Rapamycin-treated, human T cells also have reduced proliferation (89), further supporting the idea that mTORC1 is a critical regulator of T cell proliferation. Raptor-deficient T cells have reduced c-MYC and SREBP expression and activation, respectively, leading to decreased glycolysis, oxidative phosphorylation, and/or lipogenesis (16, 90).

In addition to TCR and co-stimulatory signals, amino acids also regulate mTORC1 activation to promote T cell proliferation. Depletion of select amino acids, including arginine, leucine, or tryptophan, impairs T cell proliferation (91–93). Similarly, glutamine uptake is required for efficient T cell responses, and deletion of leucine transporters, including CD98, Sla7a5, and ASCT2, reduces mTOR activation and T cell clonal expansion (29, 94–98). Interestingly, leucine and glucose import appear to be linked,

as ASCT2-deficient T cells have reduced expression of the glucose receptor, Glut1 (29). This observation may functionally link amino acid sensing to proliferation, as glucose uptake and glycolysis are intimately linked to this process (99). Collectively, these studies reveal that mTOR signaling is a crucial determinant of T cell activation.

mTOR REGULATES TRANSCRIPTIONAL AND METABOLIC PROGRAMS TO CONTROL T CELL DIFFERENTIATION

CD4$^+$ T cells

In addition to driving T cell proliferation, mTORC1 and mTORC2 also serve different roles in priming effector CD4$^+$ T cell differentiation in response to antigen, co-stimulatory, and cytokine signals. In the absence of mTOR function, T_H1, T_H2, and T_H17 polarization are all impaired (16, 54, 89, 100–102). mTORC1 activity controls T_H1 and T_H17 differentiation (100, 101). However, whether mTORC2 is also required for T_H1 generation remains controversial (100, 101). T_H2 polarization and function are severely impaired in the absence of Raptor (16), but are retained in RHEB-deficient T cells that exhibit a partial loss of mTORC1 activity (16, 101). Interestingly, although other studies link Rictor–mTORC2 to T_H2 differentiation, rapamycin treatment of *Rictor*$^{-/-}$ T cells diminishes T_H2 polarization more profoundly than Rictor deficiency alone (16, 100, 101). These data highlight the central role of mTORC1 in shaping T_H2 differentiation.

Additional work has aimed to determine the mechanisms by which mTOR links immunological signaling to effector CD4$^+$ T cell differentiation. Rapamycin treatment impairs TCR and CD28-induced T-bet and GATA3 upregulation, and also abrogates permissive de-methylation of the *Ifng* and *Il4* gene loci (103). These results may explain why deleting various mTOR-related proteins inhibits T_H1 and T_H2 differentiation. We have demonstrated that *Rptor*$^{-/-}$ CD4$^+$ T cells have profound defects in metabolic reprograming driven by the transcription factors, c-MYC and SREBP (16), which impairs the functional activation and differentiation of these cells. T_H17 differentiation is reduced in the absence of HIF-1α, a transcription factor functionally regulated by mTORC1 activity (104, 105). Interestingly, although *Rictor*$^{-/-}$ CD4$^+$ T cells do not exhibit defective T_H17 differentiation, recent studies link the mTORC2 substrate, SGK1, to the IL-23-driven generation of highly inflammatory, "pathogenic" T_H17 cells that can promote autoimmune disease development in mice (106, 107). Future work will investigate if mTORC2 regulates IL-23R signaling to facilitate this process.

Environmental cues also signal to mTOR, supporting the differentiation of CD4$^+$ T cells. It has been demonstrated that *Asct2*$^{-/-}$ T cells have reduced T_H1 and T_H17 differentiation and function as a result of reduced leucine import (29, 98). This defect is linked to attenuated TCR and CD28-induced mTORC1 activation (29). Slc7a5-deficient T cells, which have impaired amino acid transport, also have reductions in T_H1 and T_H17 differentiation (45). Moreover, S1PR1 signaling promotes T_H1 differentiation (51), while leptin receptor signaling drives T_H1 and T_H17 differentiation (108, 109). Future work will explore the detailed mechanisms by which these and other environmental signals, including additional amino acids, influence effector CD4$^+$ T cell differentiation. We describe studies implicating how mTOR signaling shapes pT_{reg} differentiation in a later section of this review.

CD8$^+$ T cells

In CD8$^+$ T cells, mTORC1 inhibition or deletion increases memory CD8$^+$ T cell formation or maintenance by regulating the expression of various transcription factors, including FoxO1, T-bet, and Blimp-1 (38, 110–113). Memory CD8$^+$ T cells may arise due to asymmetric cell division or impaired differentiation from effector CD8$^+$ T cells (81, 114). However, knocking down Raptor in activated CD8$^+$ T cells also potentiates memory functional CD8$^+$ T cell differentiation (113), and deleting TSC1 from activated CD8$^+$ T cells impairs memory differentiation and function (115). Thus, mTORC1-mediated control of memory CD8$^+$ T cell differentiation appears to be linked to defective effector to memory differentiation. mTORC1 signaling regulates CD8$^+$ T cell differentiation, in part, by controlling glycolytic and oxidative phosphorylation metabolism following IL-15 stimulation (115). However, it should be noted that IL-15-independent functions for mTOR in controlling CD8$^+$ T cell memory formation have been described (38). For instance, mTORC1 imparts control over effector versus memory T cell fate decisions by regulating the expression of NOTCH on naïve CD8$^+$ T cells (116). Thus, mTORC1 utilizes multiple mechanisms to influence effector versus memory CD8$^+$ T cell differentiation and function.

Recent data revealed a site-specific role for mTOR signaling in the generation of CD8$^+$ T cell memory. Marzo and colleagues found that rapamycin treatment enhances memory CD8$^+$ T cell differentiation in the blood and spleen, but the number of memory CD8$^+$ T cells in the lungs and peripheral lymph nodes are not affected (117). In fact, mucosal CD8$^+$ T cells isolated from the small intestine lamina propria are reduced in numbers upon rapamycin treatment, in part due to defects in T cell trafficking as discussed below. Collectively, these data indicate a critical role for mTOR in modulating tissue-specific, effector versus memory fate decisions in CD8$^+$ T cells.

In response to chronic infections, CD8$^+$ T cells become functionally impaired or exhausted (118). Kaech and colleagues recently demonstrated that Akt and mTOR signaling are impaired in CD8$^+$ effector T cells following a chronic viral infection as compared to an acute infection (119). This event leads to the FoxO1-dependent upregulation of PD-1 and promotes the survival of terminally differentiated, exhausted CD8$^+$ T cells. Signaling downstream of PD-1 antagonizes mTOR activation (120), which drives CD8$^+$ T cell exhaustion (119). Consistent with this idea, PD-1 blockade restores function in exhausted, CD8$^+$ T cells in an mTOR-dependent manner (119). Therefore, in addition to supporting CD8$^+$ T cell effector versus memory formation, the mTOR–FoxO1 axis also regulates CD8$^+$ T cell exhaustion.

mTOR MAINTAINS IMMUNE TOLERANCE BY CONTROLLING T_{reg} CELL FUNCTION AND STABILITY

Foxp3$^+$ T_{reg} cells maintain T cell homeostasis in the periphery, and their loss of function causes severe, multi-organ autoimmunity in humans and mice (121). Interestingly, mTOR signaling serves discrete functions in T_{reg} cell differentiation and function. Several groups demonstrated that T_{reg} cell differentiation is potentiated *in vitro* (called iT_{reg} cells) in the presence of rapamycin (54, 122–128). An inhibitory role for mTOR in the generation of iT_{reg} cells was further supported using *Mtor*$^{-/-}$ T cells (54), with mTORC1 and mTORC2 serving functionally redundant roles in

suppressing iT_{reg} differentiation (54, 101). Likewise, HIF-1α deficiency enhances T_{reg} cell differentiation (104, 105). However, the functional capacities of *Mtor*$^{-/-}$ or rapamycin-expanded T_{reg} cells require further investigation, as the source of the T_{reg} cells used in the *in vitro* suppression assays were not a highly purified population of Foxp3^{+} T_{reg} cells. The *in vivo* suppressive activity of these cells also remains largely unexplored, although rapamycin-expanded, human T_{reg} cells are functional in a xenograft transfer model (129).

Regulatory T cells have high, basal levels of mTOR signaling compared to their naïve T cell counterparts (39, 48). However, the proper threshold of mTOR signaling is critical to support their suppressive function *in vitro* and *in vivo*. We recently demonstrated that Raptor-deficient T_{reg} cells lose suppressive activity *in vitro* and *in vivo*, the latter of which contributes to rampant autoimmunity and lethality in mice (39). Mechanistically, Raptor-mTORC1 signaling is linked to cholesterol biosynthesis and lipid metabolism, processes that are important to support the expression of the T_{reg} cell effector molecules, ICOS and cytotoxic T lymphocyte antigen (CTLA)-4. These effects are not observed in T_{reg} cells lacking Rictor, and combined loss of Raptor and Rictor partially restores the suppressive function of T_{reg} cells *in vitro* and *in vivo* (39). Thus, loss of mTORC1, but not mTORC2, activity is linked to T_{reg} cell dysfunction.

While these studies show that loss of mTORC1 activity is deleterious to T_{reg} cell function, excessive mTOR signaling within T_{reg} cells also compromises their function and affects their stability. TSC1-deficient T_{reg} cells are impaired in their ability to suppress inflammatory responses, as they lose Foxp3 expression and acquire T_H17 cell effector-like functions *in vitro* and *in vivo* (78). Consistent with this study, recent work demonstrated that patients with autoimmune diseases have elevated mTOR activation within their T_{reg} cells (10). Although they proliferate more robustly following IL-2 stimulation, *Pten*$^{-/-}$ T_{reg} cells appear to retain their suppressive capacity *in vitro* and can suppress colitis development *in vivo* (59). However, the role of PTEN in T_{reg} cells has not been specifically addressed using a conditional deletion model. Thus, distinct negative regulators of mTOR activity appear to serve different functions in T_{reg} cells.

Several pathways have mechanistically been shown to modulate mTOR activity within T_{reg} cells to regulate their proliferation, differentiation, and function. Leptin receptor signaling restrains TCR and/or IL-2 stimulation-induced T_{reg} proliferation *in vitro* (48, 130), suggesting that leptin levels may be a critical factor influencing T_{reg} cell proliferation *in vivo* (131). Maintenance of Foxp3 expression is required for T_{reg} suppressive function (132). Transient TCR stimulation drives PI3K–Akt–mTOR signaling that antagonizes Foxp3 expression (133), and rapamycin treatment enhances Foxp3 expression by modulating DNA methylation within the *Foxp3* locus (103). Through multiple mechanisms, T_{reg} cells can modulate amino acid availability within a microenvironment (92, 121, 134). Interestingly, mTOR inhibition and amino acid deprivation synergize with TGF-β signaling to augment Foxp3 expression *in vitro* (91, 92). Finally, S1PR1 signaling to mTORC1 restrains T_{reg} differentiation in the thymus and periphery, and limits their suppressive function *in vitro* and *in vivo* during homeostasis and inflammation (50, 51).

mTOR REGULATES T CELL TRAFFICKING

After an infection occurs, chemokine and adhesion receptors localize T cells to the proper anatomical location. The adhesion receptor CD62L and chemokine receptors, CCR7 and S1PR1, allow T cells to enter and be retained in peripheral lymph nodes such that T cell activation may occur (49, 135). As with T cell development and activation, mTOR signaling is also a critical regulator of T cell trafficking. PI3K or mTORC1 inhibition in activated CD8^{+} T cells reduces IL-2-induced downregulation of CCR7, CD62L, and S1PR1 expression (136), which causes these cells to traffic to lymph nodes (34). By contrast, the downregulation of these molecules occurs more efficiently in the absence of PTEN or TSC1 (43, 115, 136, 137). These trafficking defects may partially account for why rapamycin treatment enhances and TSC1 deficiency suppresses memory CD8^{+} T cell differentiation (34, 115, 136). Although the precise mechanisms by which mTOR signaling regulates trafficking are not known, mTOR modulates the expression of Kruppel-like factor 2 (KLF2) and HIF-1α, two transcription factors that modulate the expression of lymph node homing receptors (19, 136). Further, mTORC2 may inhibit FoxO1 function by enhancing Akt activity, and FoxO1 transcriptional activity modulates the expression of lymph node homing receptors (137). Finally, mTORC1 activity induces T-bet expression (34), which drives CXCR3 upregulation and subsequently localizes T cells to sites of infection (138, 139). Thus, mTOR activity regulates T cell trafficking via multiple mechanisms (**Figure 4**).

Recent work also demonstrates a role for mTOR in T cell trafficking to non-lymphoid tissues. Trafficking into the gut-associated mucosa is regulated by CCR9, the $\alpha_4\beta_7$ integrin, and CD103 (140). In CD8^{+} T cells, rapamycin treatment suppresses the expression and/or function of these molecules, leading to a severe reduction in these cells within mucosal sites (117). Similarly, knocking down mTOR within activated CD8^{+} T cells also reduces trafficking to the small intestine. Although it was not mechanistically determined how mTOR controls mucosal site homing, the retinoic acid receptors (RARs) induce CCR9 and $\alpha_4\beta_7$ integrin

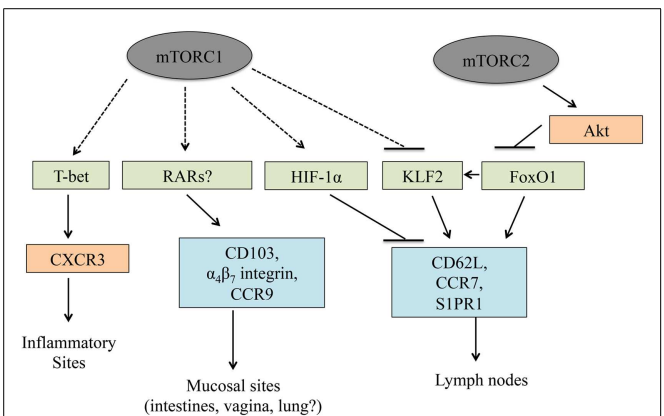

FIGURE 4 | T cell trafficking is linked to mTOR. mTORC1 and mTORC2 control T cell trafficking by regulating the expression and/or functional activation of multiple transcription factors. In this manner, mTOR signaling regulates trafficking into inflammatory sites, lymph nodes, and mucosal sites.

expression in activated T cells (141, 142). As T_{reg} cells, T_H17 cells, and iNKT cells play pivotal roles in gut-associated lymphoid reactions (143, 144), future work will need to explore how mTOR inhibition or hyper-activation influences trafficking to mucosal sites within these cell lineages.

CONCLUDING REMARKS

Current work has highlighted the critical role the environmental sensor mTOR plays in T cell biology. mTORC1 and mTORC2 both support thymocyte development, but integrate distinct and overlapping signals and impart discrete functions to facilitate this process. In contrast to thymocytes, mTORC1 is the dominant regulator of the functional activation and differentiation of conventional T cells in the periphery. mTORC1 activation is critical for clonal expansion, effector $CD4^+$ T cell differentiation, and T_{reg} cell function, while mTORC2 also contributes to these processes but with limited effects. However, further work is needed to determine the role mTORC1 and mTORC2 serve in the induction of site-specific immune responses, including the generation of T_{FH} cells and tissue-specific T_{reg} cell populations, the latter of which play critical functions in dampening immune responses in mucosal sites, adipose tissues, and tumors (121, 145).

From a clinical perspective, it will be critical to determine the impacts of mTOR inhibition on the specific immunity to pathogens, tumors, and auto-antigens. Hyper- or hypo-activation of mTOR has a profound impact on T cell development and activation, so these investigations will provide insight into how rapamycin, its rapalogs, and other next generation mTOR inhibitors will influence localized and systemic immune responses in different disease settings. Given the intricate link between mTOR function and T cell fate decisions, it is feasible that one could modulate mTOR activation within specific inflammatory sites and/or immune cell types to modulate the immune response in states where both mTOR and T cells are dysfunctional. These studies will be key toward determining if mTOR suppression in T cells is a viable target for treating autoimmunity, cancers, and infectious diseases, or for boosting memory $CD8^+$ T cell responses to enhance vaccine efficacy.

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The role of fatty acid oxidation in the metabolic reprograming of activated T-cells

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Activation represents a significant bioenergetic challenge for T-cells, which must undergo metabolic reprogramming to keep pace with increased energetic demands. This review focuses on the role of fatty acid metabolism, both *in vitro* and *in vivo*, following T-cell activation. Based upon previous studies in the literature, as well as accumulating evidence in allogeneic cells, I propose a multi-step model of *in vivo* metabolic reprogramming. In this model, a primary determinant of metabolic phenotype is the ubiquity and duration of antigen exposure. The implications of this model, as well as the future challenges and opportunities in studying T-cell metabolism, will be discussed.

Keywords: T-cell metabolism, fatty acid oxidation, oxidative phosphorylation, reactive oxygen species (ROS), AMP-activated protein kinase, graft-versus-host disease, *in vivo* models

INTRODUCTION

Activation precipitates a dramatic change in T-cell physiology. Upon stimulation, T-cells increase their DNA replication, synthesize cytokines, and upregulate multiple signaling pathways (1, 2). Proliferation increases exponentially, with stimulated cells dividing as frequently as every 4–6 h at the height of an immune response (3, 4). The energetic requirements for these new tasks dictate that T-cells must undergo metabolic reprograming in order to generate sufficient biomass and produce adequate adenosine triphosphate to meet the increased metabolic demands (5).

In recent years, increasing attention has focused on the metabolic pathways adopted by T-cells following activation. Many fine contemporary reviews highlight the relationship between metabolic phenotype and signal transduction (6, 7), T-cell differentiation (8, 9), and T-cell function (5, 10). Other reviews stand as thorough summaries on overall T-cell metabolism, and the reader is encouraged to seek out these important works (11). This review will focus on the use of fatty acid oxidation (FAO) by activated T-cells, both *in vitro* and *in vivo*, and suggest a possible connection between the environment present during activation and adoption of this alternative metabolic pathway. To place the findings on FAO into a contextual framework, I will begin by briefly reviewing the role of other metabolites, including glucose and amino acids, in T-cell metabolism.

THE NECESSITY OF GLYCOLYSIS AND AMINO ACIDS

Early studies demonstrated increased rates of both glycolysis and lactate production during mitogen activation of rat thymocytes, suggesting a prominent role for glucose metabolism during *in vitro* T-cell stimulation (12). Following activation, T-cells increase multiple steps in glucose metabolism, including upregulation of the glucose transporter Glut1, in a highly regulated process that is at least partially dependent upon signaling through the co-stimulatory molecule CD28 (13–15). Failure of T-cells to sufficiently increase glucose metabolism decreases both proliferation and cytokine production, while overexpression of a transgenic Glut1 receptor increases cytokine production and improves T-cell survival (16, 17).

Glutamine metabolism is also requisite during T-cell activation and limiting glutamine in the culture media decreases proliferation and cytokine production in mitogen-stimulated lymphocytes (18). Studies on purified populations of T-cells confirmed the importance of glutamine uptake during *in vitro* stimulation and implicated a role for CD28 in maximizing glutamine uptake (19). In addition, inflammatory CD4 T-cell responses depend on glutamine uptake through expression of the amino acid transporter Slc1a5 (20), and absence of Slc1a5 decreases the percentage of IFN-γ^+ T-cells responding to *Listeria monocytogenes* infection. Similarly, the transcription factor Myc plays a pivotal role in directing glutamine into obligate biosynthetic pathways and facilitates the initial proliferative burst (21). Thus, both glucose and glutamine appear indispensable for early events in T-cell metabolic reprograming.

In addition to glutamine, T-cells require access to other amino acids for proliferation and survival. Expression of the bidirectional glutamine/leucine transporter Slc7a5 is an integral event in early T-cell activation and absence of this receptor decreases T-cell responses both *in vivo* and *in vitro* (22). The importance of this receptor is intriguing, given that leucine is a necessary component of T-cell activation and that higher glutamine levels facilitate leucine import through simultaneous glutamine export (23). Therefore, a large role for glutamine may simply be to provide an intracellular gradient to support transport of other amino acids. This hypothesis is supported by the finding that glutamine transporter deficiency can be overcome through increasing concentrations of leucine. Additional data suggest that glutamine transport may even initiate metabolic adaptation, as absence of Slc1a5 in T-cells blunts expression of other metabolic mediators including both Glut1 and CD71 (22).

In addition to leucine, T-cells also depend upon tryptophan to execute full effector function. Suppressed T-cell responses are observed when antigen presenting cells contain indolamine 2,3-dioxygenase (IDO), an enzyme that catabolizes tryptophan (24, 25). The importance of the IDO pathway has been demonstrated in multiple immunogenic processes, including fetal tolerance during pregnancy, bone marrow transplantation, antitumor responses, and autoimmunity (26). In addition, kynurenine, a tryptophan catabolite, induces regulatory T-cell generation (T_{reg}) through its action on the aryl-hydrocarbon receptor (21). Thus, T-cell responses can be modulated by both decreased levels of a nutrient (tryptophan) and the actions of its metabolic derivative (kynurenine).

NUTRIENT REGULATION OF T-CELL DIFFERENTIATION

Other nutrients also influence T-cell differentiation and function. Short-chain fatty acids, such as propionate and butyrate, are generated via fermentation by intestinal bacteria and intestinal levels of these fatty acids also modulate T_{reg} formation (19, 27, 28). Similar to kynurenine, propionate and butyrate likely drive T_{reg} formation through specific intestinal T-cell nutrient receptors, but the precise mechanism has yet to be confirmed. In a similar way, $V\alpha9^{+}V\delta2^{+}$ gamma-delta T-cells selectively respond to the microbial metabolite (E)-4-hydorxy-3-methyl-but-2-enyl pyrophosphate (29). High salt concentrations also affect T-cell function and drive CD4 T-cells toward a Th17 phenotype both *in vivo* and *in vitro*. This is clinically relevant because dietary increases in salt worsen the severity of experimental autoimmune encephalomyelitis in murine models (30, 31). Salt sensitivity in Th17 cells occurs via increased expression of serum glucocorticoid receptor-1, a protein, which governs sodium homeostasis in multiple cell types (32). Thus, extracellular nutrients not only help meet increased energy needs of activated T-cells, but may also dictate their differentiation and effector status.

THE ROLE OF FATTY ACID OXIDATION

Oxidation of fat, in addition to the catabolism of glucose and glutamine, was first implicated as an energy source in unstimulated lymphocytes (33), although most studies suggest that naïve T-cells require only a minimal rate of metabolism to meet their bioenergetics needs (5, 34). A role for FAO in other subsets first came from work in T-cells bearing a deletion of TNF receptor associated factor 6 (TRAF6). CD8 T-cells deficient in TRAF6 are unable to form memory cells in response to infection with *L. monocytogenes* and when taken *ex vivo*, decrease rates of β-oxidation (35). Furthermore, indirect activation of AMP-activated protein kinase (AMPK), a cellular energy sensor and controller of FAO (36–38), increased CD8 memory T-cell generation and improved survival in a lethal tumor model. Subsequent *in vitro* work demonstrated that IL-15, an important cytokine in memory T-cell generation, upregulates expression of carnitinepalmitoyltransferase 1a (CPT1a), the rate limiting enzyme in FAO (39). These studies suggest a subset specific role for FAO in the generation of CD8 memory T-cells.

CD4 T-cells cultured *in vitro* also exhibit a subset specific dependence on FAO. T-cells differentiated *in vitro* toward Th1, Th2, or Th17 profiles adopt a glycolytic phenotype, consistent with earlier findings on T-cell metabolism (13, 14). In contrast, T_{regs} generated *in vitro* increase lipid oxidation and phosphorylate AMPK. Furthermore, *in vitro* blockade of FAO with the CPT1a inhibitor etomoxir disrupts T_{reg} generation and *in vitro* supplementation with fatty acids supports T_{reg} function (17). *In vivo* administration of metformin increases both the percentage and total number of T_{reg} during a murine model of asthma (17).

Together, these data support a mandatory role for FAO in both IL-15 driven CD8 T-cell responses and in the induction of *in vitro* generated T_{regs}. Furthermore, metformin administration, which indirectly activates AMPK, increases both T_{reg} and memory CD8 T-cells, and could indicate a role for AMPK in controlling FAO in these cell types (17, 35). This notion is supported by the fact that CD8 T-cells deficient in AMPKα1 mount inferior memory T-cell responses following *L. monocytogenes* infection (40). It remains unclear, however, exactly how metformin increases T_{reg} and CD8 memory T-cells and the extent to which AMPK controls pathways of T-cell metabolism beyond FAO (41–43).

Metformin is a direct inhibitor of Complex I of the electron transport chain (44) and through inhibition of oxidative phosphorylation can indirectly accelerate glycolysis (45). However, it is unlikely that increased glycolysis drives memory T-cell formation, as glycolytic inhibition has already been shown to increase CD8 memory T-cell generation (46). However, the rapid recall response of memory T-cells requires an imprinted glycolytic potential (47), suggesting that the transition from memory to effector phenotype depends upon glycolysis and is, therefore, potentially influenced by the indirect effects downstream of metformin. Further clarity on the direct role of AMPK in driving FAO and will be gained using more selective inhibitors of AMPK and genetically deficient animal models (43, 48).

One of the key challenges in studying immune cell metabolism *in vitro* is the relevance of experimental systems to the environmental conditions encountered *in vivo* (10, 49). Standard culture concentrations differ greatly from physiologic values, including higher concentrations of glucose (three- to fivefold higher than the standard serum glucose of 5 mM), glutamine (eightfold higher than serum levels), and oxygen (21% in culture compared to 2–5% *in vivo*) (50). Changes in these environmental variables can affect both a cell's function and metabolic response (51). For example, decreased glucose availability modulates both oxygen consumption and metabolic transcription factors during human CD4 T-cell activation (52). Hypoxia reduces proliferation and cytokine production and promotes glycolysis (53, 54). Thus, the metabolic phenotype adopted by a T-cell following *in vitro* stimulation may be very different from the phenotype adopted by T-cells activated under physiologic conditions *in vivo* (55).

One attractive approach to this challenge is to study metabolic reprograming in lymphocytes activated *in vivo* during graft-versus-host disease (GVHD). During GVHD, alloreactive donor T-cells respond robustly to the presence of host antigens, leading to marked proliferation, destruction of host tissues, and profound inflammation (56–58). Allogeneic T-cells taken directly from GVHD animals demonstrate a 2.5-fold increase in oxygen consumption (a surrogate for oxidative phosphorylation) and a modest increase in the expression of Glut1 (59). Increased oxidative metabolism during GVHD is consistent with data from patients with systemic lupus erythematosus, where isolated T-cells increase

their mitochondrial mass by 50% and expand their mitochondrial membrane potential by 20%. Similarly, peripheral blood mononuclear cells from these patients increase oxygen consumption by 50% compared to healthy control cells (60, 61).

Recent studies suggest a direct role for FAO in effector T-cells during GVHD. Levels of acylcarnitines, necessary intermediates in the oxidation of fat (62), increase 10-fold or more in allogeneic T-cells by day 7 post-transplant (59). In addition, effector GVHD T-cells (characterized by their $CD44^{hi}$, $CD62^{Lo}$ phenotype) increase fatty acid transport, upregulate levels of CPT1a and CPT2, and increase their rates of FAO *ex vivo* (63). Treatment of allogeneic cells with etomoxir selectively decreases their proliferation *in vitro*, while etomoxir administration *in vivo* decreases both total donor T-cell numbers and the severity of GVHD (63). In contrast, T-cells proliferating in a homeostatic fashion after transplantation, and those responding to cellular immunization, minimally increase fatty acid transport and display no sensitivity to etomoxir (63). Thus, FAO appears to be a specific metabolic adaptation in effector T-cells proliferating in response to large quantities of antigen. This is supported by findings in an experimental autoimmune encephalomyelitis model, where etomoxir blockade of FAO decreased disease severity, limited demyelination, and reduced effector cytokine production (64).

Consistent with an increase in oxidative phosphorylation, allogeneic T-cells also increase generation of reactive oxygen species (ROS) (59). Increased ROS likely result from increased mitochondrial membrane potential ($\Delta\psi m$), which prolongs the half-life of reactive intermediates in the electron transport chain, leading to increased leak of single electrons from the intra-mitochondrial space and subsequent formation of ROS (65). The increased ROS observed in allogeneic T-cells is also consistent with data from patients with systemic lupus erythematosus, where T-cells exhibit both hyperpolarization of the $\Delta\psi m$ and increased ROS (61). In addition, increases in ROS and oxidative phosphorylation can be therapeutically targeted, as modulators of complex V of the electron transport chain mitigate the severity of GVHD without affecting homeostatic reconstitution (59).

The increased glycolysis in T-cells during GVHD is modest compared to the level of glycolysis and glucose uptake observed during *in vitro* activation (14, 55). Calculations based upon O_2 consumption suggest a larger contribution from oxidative metabolism toward total energy production in allogeneic T-cells (66). This disparity might be accounted for by differences between *in vitro* and *in vivo* conditions, as described earlier (52). However, this explanation fails to account for situations where *in vivo* effector T-cells do not upregulate fatty acid transport following cellular immunization or increase mitochondrial mass following infection, as might be expected from cells with increased oxidative metabolism (39, 63). To unify the disparate results both between *in vitro* and *in vivo* conditions, and across different *in vivo* scenarios, I propose a multi-step model of metabolic reprograming in T-cells (**Figure 1**), where a primary determinant of metabolic phenotype is both the duration and degree of environmental stimulation present at the time of analysis.

In setting up this model, it is important to note that T-cells during GVHD do not increase fatty acid transport until their fifth division, which occurs on approximately day 3 post-transplant (63). Thus, early events in T-cell activation, even in the presence of significant antigen, do not require additional fatty acids. Instead, early during a graft-versus-host (GVH) response, T-cells likely utilize glycolysis and glutaminolysis to meet their short-term energy needs (16, 21). This idea is supported by data obtained following *in vivo* administration of the superantigen staphylococcal enterotoxin B (SEB), where SEB sensitive $CD4^+$, $V\beta8^+$ cells undergo a 15-fold increase in glycolysis 48 h post-administration of SEB (21). Initial dependence on glycolysis also explains the early *in vivo* sensitivity of T-cells to the glycolysis inhibitor 2-deoxyglucose (67).

Later in the response (i.e., after 4–5 cell divisions), robustly activated T-cells require additional reprograming to keep pace with the ongoing demands of persistent activation (68). This reprograming includes increased fatty acid uptake, upregulation of oxidation enzymes and co-activator molecules, moderation in the rate of glycolysis, and adoption of FAO with a concomitant rise in ROS. This view is consistent with effector T-cells maintaining oxidative phosphorylation following activation under a variety of activating conditions (21, 39, 69). Mechanisms that drive this second metabolic transition remain undefined, but signaling through PD-1 is known to restrict glycolysis (70). In addition, AMPK promotes FAO in multiple systems and is known for its ability to act as a "cellular energy sensor" (36, 37, 71, 72). Indeed, knockout of AMPKα1 increases Glut1 expression, hexokinase levels,

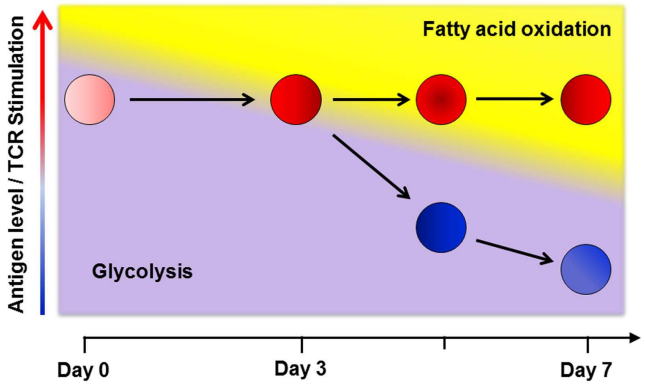

FIGURE 1 | A multi-step model of *in vivo* metabolic reprograming. Early in a graft-versus-host (GVH) response, T-cells use glycolysis, glutaminolysis and glucose oxidation to meet their short term energy needs (16, 21, 67). By day 3 post-transplant, robustly activated cells (shown in red) require additional metabolic reprograming to keep pace with ongoing energetic demands and so upregulate fatty acid oxidation (FAO) by increasing fat uptake, turning on co-activator molecules, and upregulating fatty acid oxidation enzymes. This transition comes with a concomitant rise in reactive oxygen species and a moderation in the rate of glycolysis (63, 68). In contrast, T-cells stimulated via cellular immunization, with a limited duration of antigen exposure, only transiently increase fat uptake and ROS on day 3. As antigen levels fall, stimulation decreases and cells no longer require FAO (cells shown in blue). Levels of oxidation enzymes, co-activator molecules, fat transport, and ROS levels decrease to baseline in these cells (63). Thus, despite similar $CD44^{Hi}CD62L^{Lo}$ effector profiles, the metabolic phenotype on day 7 is clearly different between robustly and transiently activated T-cells. From these data, I propose that a primary determinant of metabolic reprograming in effector T-cells is both the degree and duration (< or >3 days) of antigen exposure at the time of evaluation.

and glycolytic metabolism in purified T-cells. These observations suggest that when present, AMPK might actively dampen T-cell glycolysis (73), perhaps at the cost of promoting FAO (**Figure 2**).

Implicit in the multi-step model is the idea that continued stimulation of the T-cell receptor (TCR) drives later stages of metabolic reprograming. As antigen levels fall during the resolution of an immune response, stimulation decreases, energetic demands shrink, and effector T-cells no longer require utilization of alternative energy sources. Transgenic OT-I T-cells, when transferred into irradiated recipients that bear ovalbumin as a self-protein, markedly increase fat transport, ROS levels, and markers of oxidative metabolism. In contrast, when the same OT-I T-cells are stimulated by immunization with OVA-bearing dendritic cells, they return to baseline values of fat transport and oxidative metabolism by day 6 post-immunization (63). In these models, differences between GVHD and immunization responses were not dependent on differentiation status of the responding OT-I cells. Cells from either environment had similar $CD44^{Hi}CD62^{Lo}$ profiles and made equivalent amounts of IFN-gamma upon re-stimulation, consistent with the ability to separate effector function and metabolic phenotype on a per cell basis (74).

Although allogeneic T-cells only modestly increase glycolysis (59), glucose is still likely being utilized by these cells in alternative pathways. Indeed, shunting of glucose through the pentose phosphate pathway increases levels of reducing equivalents through production of NADPH and also generates building blocks for nucleic acid synthesis (75). In addition, glucose derivatives can be combined with oxaloacetate in the TCA cycle to form citrate, exported back to the cytosol via the carnitine/palmitate shuttle, converted into acetyl-CoA and then malonyl-CoA via the action of acetyl-CoA carboxylase 1 (ACC1), and eventually incorporated into *de novo* lipid synthesis. Evidence that a similar process occurs in activated T-cells comes from work using ACC1 deficient T-cells. CD8 T-cells lacking ACC1 have impaired survival following *L. monocytogenes* infection (74) and ACC1 deficiency preferentially disrupts *in vitro* differentiation of Th17 T-cells while simultaneously sparing development of T_{reg} (76). This Th17/T_{reg} dichotomy is reminiscent of studies on hypoxia-inducible factor 1-α and aryl-hydrocarbon receptor signaling, where promotion of Th17 responses occurs at the expense of T_{reg} generation (77, 78). In addition, both treatment with an ACC1 specific inhibitor and use of ACC1 deficient T-cells leads to diminished severity of EAE (76). However, in contrast to FAO inhibition, which preferentially affects antigen-activated T-cells (63), deficiency of ACC1 impacts both antigen-activated and homeostatic responses (74). This suggests that lipid synthesis, as driven by ACC1, is likely a necessity for proliferation of all T-cells.

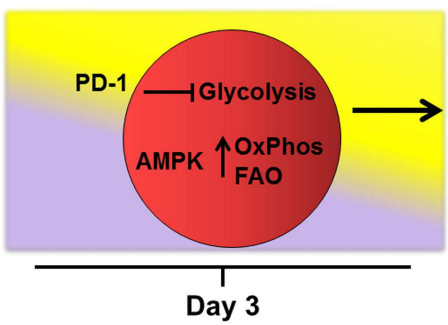

FIGURE 2 | Potential drivers of fatty acid oxidation. Mechanisms that drive transition of robustly activated T-cells toward oxidative metabolism and fatty acid oxidation remain undefined. The checkpoint molecule PD-1 is upregulated early on allogeneic T-cells and PD-1 expression tracks with levels of reactive oxygen species during GVHD (manuscript in preparation). In addition, signaling through PD-1 is known to restrict T-cell glycolysis (70). AMPK acts as a "cellular energy sensor" and may respond to increasing AMP/ATP ratios as cells proliferate beyond 4–5 cell divisions. AMPK promotes oxidative metabolism (OxPhos) and FAO in multiple systems (36, 37, 71, 72) and knockout of AMPKα1 increases glycolytic metabolism in T-cells (73). Thus, activated AMPK might simultaneously dampen T-cell glycolysis while promoting adoption of FAO during periods of persistent antigen activation.

Similar to the diversity of glucose metabolism, glutamine may, in addition to glutaminolysis, play a role as a metabolic substrate in one-carbon serine metabolism, as has been shown for proliferating cancer cells (79). Thus, as effector T-cells proliferate beyond 4–5 cell divisions with ongoing TCR stimulation, the roles of glucose and glutamine likely change, as increased fat transport feeds fatty acids into the TCA cycle. However, even fat-derived intermediates may exit the TCA cycle into alternative metabolic shunts (e.g., via the aspartate-malate shuttle) and the role of these divergent pathways during the latter stages of ongoing T-cell activation remain exciting areas for future investigation.

Given the ability of effector T-cells to reprogram their metabolic phenotype, the question arises as to whether T-cells can be pre-programed for the physiologic conditions they will encounter *in vivo*, a potential advantage when providing anti-viral or anti-tumor immunity. Recent work lends credence to this possibility. Treatment of *ex vivo* CD8 T-cells with the glycolysis inhibitor 2-deoxyglucose (2DG) increases phosphorylation of AMPK, heightens oxygen consumption, and decreases multiple markers of glycolysis in these cells. In addition, *ex vivo* treatment of cells with 2DG enhances their *in vivo* antitumor function (46). These data suggest that glycolytic inhibition *ex vivo* drives upregulation of alternative metabolic phenotypes, which then provide a subsequent selective survival advantage *in vivo*.

In contrast to allogeneic activation, T-cells responding via homeostatic proliferation minimally upregulate fatty acid transport and are not susceptible to FAO inhibition. This observation suggests that the metabolic demands of homeostatic renewal are distinct from those of T-cell activation, as has been seen in other studies of T-cell metabolism (22). These results also imply that modulating T-cell metabolism may offer a selective intervention against pathogenic cells, potentially leading to a decrease in overall immunosuppression (55, 59). From this perspective, it becomes critically important to understand at which stage of metabolic reprograming the intervention occurs. Some therapies, such as antithymocyte globulin, will eliminate all T-cells regardless of proliferation or activation status. Other treatments, like inhibition of ACC1, will affect survival of all proliferating T-cells, regardless of the stimulus (antigen-activated versus homeostatic cues) (74). Interventions that disrupt early events in TCR-activated metabolic reprograming (e.g., disruption of amino acid transport) might spare homeostatic T-cells, but will target T-cells whose metabolic

reprograming is driven through TCR stimulation (80). Finally, interventions that affect the latter stages of metabolic adaptation, such as inhibition of FAO or modulation of oxidative phosphorylation, will likely only inhibit effector T-cells responding to prolonged antigenic stimulus (63). This last form of intervention may be particularly relevant in T-cells undergoing continuous exposure to antigen (e.g., during autoimmunity and following transplantation of bone marrow or solid organs) and highlights situations that will gain the most from selective immunotherapy, as current immunosuppression for these disorders leads to significant morbidity and mortality (81–84).

CHALLENGES FOR THE FUTURE

The rapid increase in our understanding of T-cell metabolism offers exciting opportunities and presents several challenges. The majority of initial metabolic studies were performed *in vitro* and many of these paradigms and results may not reflect *in vivo* biologic reality, which needs to be addressed. In addition, metabolic adaptation is by necessity a dynamic and responsive process, and conditions both inside and outside the T-cell change dramatically from one moment to the next. Thus, a thorough view of metabolism in any model needs to incorporate data and observations from multiple time points of analysis. Third, we know very little about the molecular machinery that drives adoption of metabolic phenotypes, particularly *in vivo*. Follow-up studies will need to not only identify proteins important in T-cell metabolism, but also define how the dependence on these factors changes during the course of an immune response. The field also needs metabolic activators and inhibitors with increased specificity, both for study purposes and the potential for therapeutic intervention (43). Finally, the study of T-cell metabolism needs to expand to better include the human immune system, particularly in the context of immune-mediated disorders. Future studies in human beings will benefit both from clinically based flux analysis using labeled metabolites such as ^{13}C-glucose or ^{13}C-palmitate (85, 86) and the great variety of pathways and compounds being discovered in the field of cancer therapy (79, 87–90).

CONCLUSION

T-cell activation represents a time of significant energetic stress and cells must respond to this challenge by reprograming their metabolism to keep pace with increased metabolic demands. During a murine model of GVHD, effector T-cells increase their dependence on oxidative metabolism and FAO. Adoption of these pathways is likely due to environmental factors present at the time of T-cell recovery and analysis, including the ubiquity and duration of antigen exposure. Thus, T-cell differentiation status (e.g., memory versus effector) is not the sole arbitrator of metabolic phenotype, and our data suggest that effector T-cells will instead respond as necessary to meet the metabolic demands placed upon them, including upregulation of FAO. Future studies will determine how broadly findings on allogeneic T-cells can be applied to other models of chronic antigen exposure. Finally, these hypotheses must be tested in human immune responses, where a better understanding of T-cell metabolism might lead to enhanced vaccine strategies, improved anti-cancer responses, novel interventions against autoimmunity, and better post-transplant immunotherapy.

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Glucose metabolism regulates T cell activation, differentiation, and functions

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The adaptive immune system is equipped to eliminate both tumors and pathogenic microorganisms. It requires a series of complex and coordinated signals to drive the activation, proliferation, and differentiation of appropriate T cell subsets. It is now established that changes in cellular activation are coupled to profound changes in cellular metabolism. In addition, emerging evidence now suggest that specific metabolic alterations associated with distinct T cell subsets may be ancillary to their differentiation and influential in their immune functions. The "Warburg effect" originally used to describe a phenomenon in which most cancer cells relied on aerobic glycolysis for their growth is a key process that sustain T cell activation and differentiation. Here, we review how different aspects of metabolism in T cells influence their functions, focusing on the emerging role of key regulators of glucose metabolism such as HIF-1α. A thorough understanding of the role of metabolism in T cell function could provide insights into mechanisms involved in inflammatory-mediated conditions, with the potential for developing novel therapeutic approaches to treat these diseases.

Keywords: glucose transporter 1, PI3K, metabolism, mTOR, HIF-1α, immune activation, HIV, inflammation

INTRODUCTION

The immune system comprises specialized cell populations that are conditioned to respond rapidly and vigorously to antigenic and inflammatory signals. Most research has focused on these signals in guiding immune responses. Now emerging data indicate that cellular metabolism regulates immune cell functions and differentiation, and consequently influences the final outcome of the adaptive and innate immune response (1–4). The growth, function, survival, and differentiation of an activated immune cells depend on dramatic increases in glucose metabolism as fuel, a process that is directly regulated and has a profound impact on health and disease (1, 5–7).

Inflammatory conditions such as HIV infection results in a heightened inflammatory state that affects the availability and use of energy. This in turn influences T cell activation and functions (8, 9). Identifying the pathways that coordinate the metabolic processes during inflammatory conditions, as observed in HIV infection will potentially provide new therapeutic opportunities.

METABOLIC PROFILES OF IMMUNE CELLS DURING IMMUNE ACTIVATION

The functions of peripheral T cells are maintained and are intimately linked to metabolism. Specific effector functions are unable to proceed without the cell adopting the appropriate metabolic state (10, 11). Research into T cell metabolism has provided valuable insight into the pathways that are important for T cell fate, plasticity, and effector functions. T cells rapidly transition between resting catabolic states (naïve and memory T cells) to one of growth and proliferation (effector T cells) during normal immune responses (10, 11).

The commitment of an immune cell to a specific metabolic pathway depends on the particular function. This is evident in the subsets of CD4+ T cells where effector T cells and Th17 cells rely on aerobic glycolysis, while memory T cells and T regulatory cells (Treg) rely on fatty acid oxidation to produce energy (12). Aerobic glycolysis is also utilized for energy by activated dendritic cells, neutrophils, and pro-inflammatory macrophages (13).

The vast majority of evidence supporting the significance of metabolism in immune cell functions is derived mainly from *in vitro* and animal models. The reasons why T cells adopt specific metabolic programs and the impact this has on their function in the context of human diseases such as HIV infection remains unclear.

HOW IS GLUCOSE USED BY IMMUNE CELLS TO PRODUCE ENERGY?

Glucose is transported into T cells via the high affinity Glucose transporter 1 (Glut1), which is the major glucose transporter on T cells (14, 15). Through a rate limiting step catalyzed by hexokinase, glucose is trapped inside the cells where it is metabolized via glycolysis. During this process, each glucose molecules is broken down into pyruvate with a net production of two ATP molecules. Most non-proliferating and terminally differentiated T cells such as naïve and memory T cells completely oxidize pyruvate via the

tricarboxylic acid (TCA) cycle to generate NADH and FADH2 that fuel oxidative phosphorylation producing 36 molecules of ATP per glucose molecule. When T cells are activated, pyruvate is transformed into lactate regenerating NAD+ that subsequently engages glycolytic reactions.

It may seem counterintuitive that T cells, which have increased demand for energy would be involved in exploiting a relatively insufficient process to generate energy. Whilst glycolysis is less efficient in generating ATP than oxidative phosphorylation, it is a rapid process occurring independently of mitochondrial function. Furthermore, a widely held assumption is that the shift from oxidative phosphorylation to increased aerobic glycolysis by rapidly proliferating T cells diverts the use of glucose for macromolecular biosynthesis (16).

GLUCOSE METABOLISM IN NAÏVE AND ACTIVATED T CELLS

Upon maturation in the thymus, naive CD4+ T cells recirculate between the blood and secondary lymphoid organs. The immune quiescence of naïve T cells is accompanied by a catabolic metabolism, characterized by the breakdown of glucose, fatty acids, and amino acids to generate intermediate metabolites, which enter the mitochondrial TCA cycle (17). The interconversion of metabolites in the TCA cycle generates energy and reducing equivalents, which subsequently enter the oxidative phosphorylation pathway effectively increasing ATP production.

The quiescence of naïve T cells is interrupted upon engagement of the T Cell Receptor (TCR) by a specific antigen/MHC class II complex displayed on the surface of dendritic cells, concurrently with the recognition of costimulatory molecules by the receptor CD28. These two signals trigger T cell activation, the secretion of IL-2, cellular proliferation referred to as clonal expansion, and their differentiation into an effector phenotype. These changes in the activation status of CD4+ T lymphocytes not only require energy, but also increased demand for metabolic precursors for the biosynthesis of proteins, nucleic acids, and lipids to fuel clonal expansion and subsequent differentiation into effector cells. Therefore, efficient T cell activation requires profound changes in cellular metabolism (18, 19). In effect, energy generation through the TCA cycle and oxidative phosphorylation is interrupted and have been thought to be replaced by glycolysis, in which glucose is converted to lactate in the cytosol, even when sufficient oxygen is available to perform oxidative phosphorylation (5, 20).

The peculiar promotion of glycolysis in the presence of normal oxygen levels is referred to as aerobic glycolysis and it is also a hallmark of cancer metabolism (21, 22). Although less efficient in terms of energy production, aerobic glycolysis generates metabolic intermediates that are used in anabolic pathways required to sustain cell growth and to produce daughter cells. However, more recently the dogma that CD4+ T cells simply switch from an oxidative to glycolytic metabolism has been challenged. Cao and colleagues demonstrated that oxidative phosphorylation is strongly induced during CD4+ T cells activation (23). By comparing CD4+ and CD8+ T cells, the researchers showed that these cells utilize distinct metabolic strategies to meet their functional demands. Following activation, CD8+ T cells had a higher glycolytic flux than CD4+ T cells. On the other hand, CD4+ T cells also induced glycolysis upon activation, but had greater mitochondrial content and oxidative metabolism than CD8+ T cells. Nevertheless their observation that glycolytic inhibition by 2 deoxy-glucose (2-DG) suppressed CD4+ T cell growth, and that rotenone inhibited both CD4+ and CD8+ T cell proliferation underscores the significance of glycolysis and oxidative metabolism in T cell activation (23). It is therefore apparent that T cell activation is not accompanied merely by a switch from oxidative metabolism to glycolysis, but that both pathways are upregulated to support bioenergetic demands. This intimate interrelationship between T cell activation and metabolism led to the concept that changes in T cell metabolism are not simply a consequence of antigen-induced activation, but rather a parameter that determines T cell proliferation and fate decisions (5, 24).

SIGNALING PATHWAYS REGULATING GLUCOSE METABOLISM IN T CELLS

In activated T cells, the rapid induction of glycolysis is promoted by the increase in the activity of several enzymes and proteins, which are regulated at the transcriptional and posttranscriptional levels. Following T cell activation, Glut1, is translocated to the surface of CD4+ T cells (25–28). This occurs in response to the activation of the phosphoinositol-3 kinase (PI3K)-Akt pathway that triggers the recruitment of Glut1 from the cytoplasmic pool to the cell surface. Increased Glut1 expression and glucose uptake by activated T cells is accompanied by increased glycolysis (**Figures 1A,B**) (29). An abnormal transduction of Akt signaling was discovered among Fas-associated protein with death domain (FADD) knock out thymocytes, and was partly responsible for a decline in Glut1 expression, a corresponding decrease in glucose uptake, increased apoptosis, and reduced cell numbers (30). In addition, T cell activation accompanies induction of the mammalian target of rapamycin (mTOR) pathway. mTOR is a serine/threonine kinase that forms two multiprotein complexes, mTORC1 and mTORC2, as determined by the association with different adapter and scaffolding proteins. mTOR activation regulates a myriad of cellular functions, including growth, apoptosis, differentiation, and metabolism (31, 32). Recently, the mTOR pathway has generated enormous attention due to the regulation and differentiation of distinct T cell subsets by different mTORC complexes (7), and the considerable interest in these complexes by the pharmaceutical industry (33). Other signaling pathways that have been associated with glucose metabolism in T cells are the extracellular signal-related kinase (ERK) (34), signal transducer and activator of transcription (STAT5) (15), some MAPKinases (35), and hexokinase II (36). However, the magnitude by which these pathways regulate T cell metabolism may vary depending on the precise environmental conditions. Indeed, it is also likely that these pathways may also co-operate with the PI3K-Akt and mTOR pathways to regulate metabolic reprograming of T cells.

mTOR REGULATION OF GLUCOSE METABOLISM, T CELL ACTIVATION, AND DIFFERENTIATION

During metabolism in CD4+ T cells, activation of the mTOR by TCR/CD28 coligation interrupts catabolic metabolism by regulating fatty acid oxidation and oxidative phosphorylation (12). Concurrently, mTOR induces the transcription of many key glycolytic enzymes (37). Thus, the increase in glucose uptake due

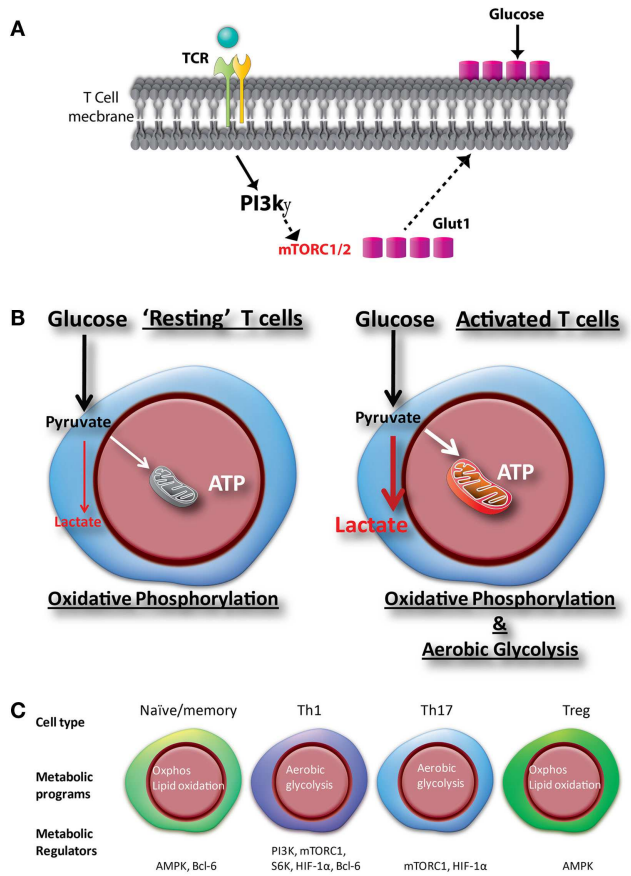

FIGURE 1 | Glucose metabolic programs regulate glucose uptake, activation, and differentiation of CD4+ T cells. **(A)** Mitogenic stimulation or engagement of the TCR complex activates the phosphoinositide 3-kinase (PI3K)-gamma (PI3Kg) subunit in CD4+ T cells. Activation of the PI3Kγ in CD4+ T cells promote Glut1 trafficking from the cytoplasm to the cell membrane and increases glucose uptake to sustain activation. **(B)** Upon activation, T cells increase glucose uptake through Glut1, which facilitate increased oxidative phosphorylation and glycolysis to sustain cell growth and proliferation. **(C)** Unique metabolic changes upon differentiation of T cells toward different subsets, regulated by various transcription factors and signaling pathways.

to Glut1 translocation, together with enhanced transcription of glycolytic enzymes results in an important increase in the glycolytic flux. The regulation of metabolic pathways by mTOR is mainly achieved through the activation of downstream transcriptional factors, such as Bcl-6 involved in the regulation of T cell immune function (38, 39), which reinforces the current paradigm that metabolism and T cell function are deeply interconnected. In particular, mTOR activation stimulates the activity of the transcription factor Myc, which plays a key role in the metabolic switch following activation by promoting expression of enzymes involved in aerobic glycolysis and other anabolic pathways (17). In addition, mTOR enhances the translation of the mRNA encoding HIF-1α. The increase in HIF-1α level also facilitates the expression of critical components of the glycolytic pathway and regulates the balance between Treg and inflammatory Th17 differentiation through direct transcriptional activation of RORγt thus promoting T cell differentiation and activation (40, 41).

METABOLIC SIGNATURES OF CD4+ T CELL LINEAGES

The development of an effective and balanced immune response is largely determined by the differentiation status (Naïve/memory) and effector profile of T cells (Th1, Th17, and Treg), and is controlled by distinct metabolic programs (**Figure 1C**). Each of these lineages has a distinctive functional property, largely determined by the production of a defined subset of cytokines (42). The lineage commitment of activated T cells is determined by the integration of multiple cues present in the immune microenvironment at the moment of activation. Interestingly, both the metabolic and immunologic programs are coordinated by the mTOR (43). Thus, mTOR activity is required for the differentiation of all the CD4+ T cell effector subsets but not for the differentiation of Tregs (44, 45). Indeed, whereas effector T cells are highly glycolytic, Tregs have a metabolism dominated by the oxidation of fatty acids followed by oxidative phosphorylation (12).

The activity of mTOR not only influences effector versus regulatory decisions, but also plays a critical role in the differentiation of the different effector T cell profiles. As follows, signaling from the mTORC1 is required for the differentiation of Th1 and Th17 but not of Th2 cells (46). Conversely, the mTOR signaling through mTORC2 is required for the differentiation of Th2 cells (46).

METABOLIC PROGRAMING OF TH1 CELLS

Th1 cells are functionally characterized by the production of IFNγ and TNF, which are of utmost importance in the induction of cell-mediated immunity against obligate intracellular pathogens, such as viruses, as well as bacteria, such as *M. tuberculosis*. Th1 cells possess a high glycolytic rate, which is paralleled by high surface expression levels of Glut1 (12). Remarkably, the glycolytic metabolism of Th1 cells dramatically influences their functionality, as evidenced by the fact that inhibition of glycolysis severely suppresses the secretion of IFNγ (6, 10). In addition to the role of aerobic glycolysis in biomass production, glycolysis and IFNγ production present one extra level of interaction. Indeed, it has been proposed that glycolytic enzymes can regulate the effector phenotype of T cells by performing non-metabolic functions. For example, the glycolytic enzyme GAPDH, if not engaged in glycolysis, can bind IFNγ mRNA post-transcriptionally, blocking the translation of this cytokine. Thus, aerobic glycolysis would be required to engage GAPDH in its metabolic functions, liberating the IFNγ mRNA for translation, thereby allowing these cells to attain full effector functions (10). Interestingly, studies conducted by Cham and Gajewski elegantly demonstrated that IFNγ, but not IL-2, production is preferentially inhibited by limiting glucose conditions (47). This further highlights the important link between glucose metabolism regulated by mTORC1, and CD8+ T cell effector functions. Although inhibition correlated with reduced phosphorylation of p70S6 kinase and eIF4E binding protein 1, surprisingly, inhibition of mTOR failed to block T cell cytokine production under their experimental conditions (47). This illustrates the complex relationship between transcriptional and post

transcriptional control of T cell effector functions, mediated by metabolic reprograming.

METABOLIC PROGRAMING OF TH17 CELLS

Th17 cells produce IL-17, IL-21, and IL-22 and are critical for the control of extracellular bacteria and mucosal immunity. Moreover, Th17 cells are important for of a number of autoimmune processes (48). Like Th1 cells, Th17 cells are highly glycolytic (12). In addition to the requirement of signaling from the mTORC1 (46), Th17 cell differentiation is critically dependent on the transcription factor HIF-1α. HIF-1α not only stimulates the glycolytic activity of Th17 cells, but also transcriptionally activates the master transcription factor RORγt (40), which subsequently directs the differentiation program of Th17 cells (49). Thus, it is therefore clear that metabolic programs can now be used to identify and classify effector T cell lineages as reviewed by MacIver and colleagues (7).

METABOLIC PROGRAMS IN DIFFERENTIATION AND EXPANSION OF Tregs

Lipid oxidation via AMPK and oxidative phosphorylation are considered the predominant metabolic programs in differentiated Tregs (7, 12). However, Neildez-Nguyen and colleagues have recently shown that higher expression of Glut1 is detected on mouse Tregs generated under hypoxic (5% O_2) culture conditions compared to those cultured under ambient oxygen levels (21% O_2) (50). Indeed following differentiation, amplification of the committed Tregs was explicitly favored by low oxygenation, and by glycolysis probably through induction of Glut1 on the cell membrane (50). This observation underscores the significance of culture conditions such as oxygen levels in regulating metabolism and thus cautions how one interprets and relate *in vitro* metabolic activity to those in humans. The significance of these distinctions is confirmed by the expression and stability of HIF-1α, which is highly dependent on the level of oxygenation in the cellular environment. Augmented Glut1 expression, mediated by HIF-1α, is observed in response to low O_2 levels in several cell types including CD4+ T cell (9). HIF-1α transcriptionally activates genes encoding glucose transporters, and rate limiting enzymes involved in glycolysis, and therefore plays a significant role in T cell differentiation and functions (39, 51).

T CELL METABOLISM IN INFLAMMATORY DISEASES

Despite the overwhelming evidence suggesting that specific metabolic alterations is associated with T cell functions and differentiation; how these metabolic changes influences immune functions in human diseases has only recently been examined. Studying Glut1 levels on immune cells, Palmer and co-workers have shown that increased glycolytic metabolism in CD4+ T cells is associated with abnormally high levels of immune activation, and low CD4+ T cell count in HIV-infected persons (52), at least in part due to "metabolic exhaustion" of these cells. Indeed, recent investigations have demonstrated that Glut1 is a CD4+ T cell activation marker essential for cell growth and proliferation, and HIV infection *in vitro* (9, 53). It is unknown whether other inflammatory conditions such as obesity, diabetes, cardiovascular diseases, and rheumatoid arthritis can impact cellular metabolism of T cells and other immune cells. In the context of HIV infection, an established chronic inflammatory disease, increased glucose metabolism in inflammatory monocyte subsets was associated with elevated levels of markers of inflammation (54, 55).

As discussed above, following activation and differentiation, the pro-inflammatory CD4+ T cell subsets are distinguished from the anti-inflammatory CD4+ Tregs based on their metabolic signatures. Thus, intense investigations are now focused on the hypothesis that elevated glycolysis is a hallmark of inflammatory cells. Indeed, inhibition of glycolysis by rapamycin has been shown to facilitate the generation of murine naturally occurring CD4+CD25+Foxp3+ Tregs *in vitro*, which were able to prevent allograft rejection *in vivo* (56), illustrating the link between T cell differentiation, metabolism, and immunity.

Recently, the role of oxidative stress, a hallmark in several inflammatory conditions has been discussed in the framework of metabolism in immune cells (1). In inflammatory macrophages, reactive oxygen species (ROS) have been implicated in increased Glut1 expression and glycolysis, mediated in part by NF-kB signaling (1). Data regarding the role of ROS in T cell metabolism are sparse; however, upon T cell activation, mitochondrial ROS are generated within minutes, and at low levels is associated with cellular proliferation (57, 58). Therefore, a plausible model in the context of T cells is that ROS induce HIF-1α by activating PI3K/mTOR and or NF-kB-linked signaling to upregulate metabolic pathways that facilitate T cell expansion and proliferation (59). Another important consideration is the interaction between T cells and other immune cells such as monocytes and macrophages. Inflammatory mediators produced by activated monocytes and macrophages are potential sources of activating stimuli for T cells (54), thus a thorough understanding of the shared metabolic checkpoints by which diverse inflammatory cues and oxidative stress modulate metabolic programing will provide important insight into combined approaches to target cellular metabolism in T cells. Moreover, the prominence of immune cells in controlling inflammatory-associated inflammation such as obesity has now gained considerable attention (60). The exciting advances in immunometabolism may provide new opportunities to develop novel interventions for the treatment of inflammatory and metabolic diseases.

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The environment of regulatory T cell biology: cytokines, metabolites, and the microbiome

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Regulatory T cells (Tregs) are suppressive T cells that have an essential role in maintaining the balance between immune activation and tolerance. Their development, either in the thymus, periphery, or experimentally *in vitro*, and stability and function all depend on the right mix of environmental stimuli. This review focuses on the effects of cytokines, metabolites, and the microbiome on both human and mouse Treg biology. The role of cytokines secreted by innate and adaptive immune cells in directing Treg development and shaping their function is well established. New and emerging data suggest that metabolites, such as retinoic acid, and microbial products, such as short-chain fatty acids, also have a critical role in guiding the functional specialization of Tregs. Overall, the complex interaction between distinct environmental stimuli results in unique, and in some cases tissue-specific, tolerogenic environments. Understanding the conditions that favor Treg induction, accumulation, and function is critical to defining the pathophysiology of many immune-mediated diseases and to developing new therapeutic interventions.

Keywords: regulatory T cells, FOXP3, cytokines, metabolites, microbiome, plasticity, environment, immune regulation

INTRODUCTION

Regulatory T cells (Tregs) are a suppressive subset of $CD4^+$ T helper (Th) cells important for the regulation of immune responses. The best-characterized Tregs are defined by expression of the transcription factor forkhead box protein 3 (FOXP3) and demethylation of the Treg-specific demethylated region (TSDR) in the FOXP3 locus. Demethylation of this element is thought to be crucial to maintain the stable, high expression of FOXP3 necessary for lineage stability and suppressive function (1, 2). Additional Treg markers include constitutive expression of the high-affinity IL-2Rα chain (CD25) and cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) (3), along with low expression of the IL-7Rα chain (CD127) (4, 5). $CD4^+CD25^+FOXP3^+$ Tregs can be divided into two main types: thymically derived Tregs (tTregs) and peripherally derived Tregs (pTregs) (6). Although it is difficult to distinguish between tTregs and pTregs phenotypically, both are thought to have an essential role in immune regulation (7).

Because of their immunoregulatory function, Tregs are an attractive therapeutic target in many different immune-mediated diseases, including transplantation, autoimmunity, and autoinflammation (8). An emerging concept is that Tregs are functionally specialized to their local environments (9), with the local milieu of cytokines, metabolites, and catabolites having major effects on the phenotype and function of these cells. In this review, we discuss current knowledge on how environmental factors affect Treg development, maintenance, and function, focusing on key recent findings in the area of cytokines, metabolites, and the microbiome.

CYTOKINES

THE ROLE OF CYTOKINES IN tTreg DEVELOPMENT IN THE THYMUS

Development of tTregs in the thymus is critically dependent on signals from the T cell receptor (TCR), CD28, and cytokines. Of particular importance are cytokines that signal via the common γ chain (γ_c), (10, 11), a topic that has been extensively reviewed (12, 13) (**Table 1**). Although most data suggest that IL-2 provides the essential signal to $CD25^+FOXP3^-$ single positive tTreg precursors to differentiate into $FOXP3^+$ cells, in the absence of IL-2, IL-15 provides a compensatory mechanism (14). In addition, a recent report found that $CD25^-FOXP3^+$ precursors have a specific requirement for IL-15 signaling to develop into tTregs *in vitro* and *in vivo* (15). Notably, while a complete absence of signaling from γ_c cytokines leads to a total lack of Tregs (16), in IL-2/IL-15-deficient mice a few Tregs remain (17). These findings suggest there other cytokines that signal through γ_c that can partially substitute for IL-2 and IL-15 in instructing tTreg development. An unanswered question is what cells in the thymus make IL-2, and/or other γ_c cytokines, and under what conditions? As dendritic cells (DCs) have been shown to make IL-2 (18) and are present in the human thymic medulla in close proximity to developing tTregs (19) they are an obvious candidate, but this has yet to be experimentally investigated.

In addition to γ_c cytokines, TGF-β also has a critical role in tTreg development. In mice, thymocyte apoptosis leads to production of TGF-β by thymic macrophages, DCs, and epithelial cells, leading to TGF-β-induced FOXP3 expression and tTreg differentiation (20). Interestingly, in mice, this apoptosis in the thymus only occurs after birth, providing an explanation for the long-standing finding that murine tTregs only begin to develop 3 days after birth (21). How this finding relates to tTreg development in humans is unknown, but neonatal humans clearly have tTregs (22), so presumably this process occurs long before birth. Although the relative importance of IL-2 versus TGF-β in tTreg differentiation versus survival is a subject of much debate, both of these cytokines are clearly important for this lineage and understanding the biology of this

Table 1 | Summary of cytokines that influence Tregs.

Treg stage	Cytokine	Species	Cytokine function	Reference
Thymic development	IL-2, IL-15	Mouse	Drives development of tTregs by inducing FOXP3 via STAT5	(14, 16, 210)
	IL-7	Mouse	Promotes development of Tregs in absence of IL-2/IL-15	(17)
	TGF-β	Mouse	Induces FOXP3 expression	(20, 211)
Peripheral development	IL-2	Mouse	Critical for TGF-β-induced pTreg development	(23, 212)
			Decreases IL-6R expression, prevents Th17 differentiation	(213)
	TGF-β	Mouse	Induces FOXP3 expression in naïve $CD4^+$ T cells *in vitro* and *in vivo*	(24, 214)
		Human	Induces FOXP3 expression in naïve $CD4^+$ T cells *in vitro*	(31, 215, 216)
	TNF-α	Mouse	Impairs TGF-β-induced differentiation of pTregs	(92)
Homeostasis	IL-2	Mouse	Up-regulates pro-survival proteins	(51, 52)
			Involved in pTreg homeostasis	(14)
			Maintains Treg GATA3 expression, which suppresses T-bet and RORγt induction	(54–56)
		Mouse/ Human	Controls size of Treg pool *in vivo*	(53)
			Induces and stabilizes FOXP3, regulates key Treg-signature molecules	(217–219)
	IL-7	Mouse	Promotes homeostasis of $IL\text{-}7R\alpha^+$ memory Tregs in the skin	(57)
	IL-15	Mouse	Promotes homeostasis of $IL\text{-}15R\beta^+$ memory Tregs accumulating with age	(58)
	IL-33	Mouse	Induces proliferation of colonic $ST2^+$ Tregs, increases TGF-β-induced differentiation of $ST2^+$ Tregs *in vitro*	(84, 85)
			Induces $CD4^+$ $FOXP3^+$ Treg proliferation *in vivo*	(81–83)
Function	TNF-α	Human	Reduces FOXP3 mRNA and protein expression levels in Tregs	(89, 90)
			TNF-α-membrane bound: reduces suppressive capacity of Tregs	(91)
		Mouse	Impairs Treg function	(89, 92, 220)
			Augments Treg function and proliferation	(93, 97)
Differentiation	IL-4	Mouse	Induces Th9 differentiation in presence of TGF-β	(221, 222)
	TGF-β + IL-1β, IL-6, IL-21, IL-23, TNF-α	Mouse	Induces Th17 differentiation and maintains Th17 cells	(220, 223–225)
	TGF-β + IL-1β, IL-6, IL-21, IL-23	Human	Induces Th17 differentiation and IL-17 secretion	(225–228)
	IL-23	Mouse	Inhibits Treg differentiation *in vitro* and Treg accumulation in gut	(86, 87)
Th-like Tregs/ex-Tregs	IL-6	Mouse	Induces IL-17 secretion and conversion of Tregs to Th17	(101)
	IL-1β, IL-2, IL-6, IL-15, IL-21, IL-23	Human	Combinations of these cytokines induce IL-17 secretion by Tregs	(102, 103, 105, 107, 112)
	IL-12	Human	Induces expression of T-bet, CXCR3, and IFN-γ production in Tregs	(114–116)
	IL-12, IL-27, IFNγ	Mouse	Induces expression of T-bet, CXCR3, and IFN-γ production in Tregs	(106, 109, 110)

system in humans will be key to developing therapies to boost tTreg development *in vivo*.

THE ROLE OF CYTOKINES IN pTreg DEVELOPMENT IN THE PERIPHERY

The appropriate cytokine milieu is also a critical factor for the development of pTregs. In mice, both TGF-β and IL-2 are required to drive the conversion of $CD4^+CD25^-FOXP3^-$ naïve T cells into $CD4^+CD25^+FOXP3^+$ pTregs (11, 23–25). However, the final outcome of TGF-β signaling is highly influenced by other surrounding cytokines. For example, anti-inflammatory conditions augment the effects of TGF-β, potentiating pTreg development (26). Conversely, pro-inflammatory cytokines (IL-1β, IL-6, IL-21, IL-23, and/or TNF-α) counteract TGF-β-induced FOXP3 expression and instead drive Th17 cell development by enhancing expression of retinoid-related orphan receptor γt (RORγt), the master Th17 lineage transcription factor (27).

Because of their potential application as a cell-based therapy, many groups have explored the cytokine combinations that can drive the differentiation of $FOXP3^+$ Tregs *in vitro* (iTregs) from naïve human $CD4^+$ T cells. Early evidence suggested that, as for mice, TCR stimulation in the presence of both TGF-β and IL-2 induced FOXP3 expression. However, the interpretation of these data became difficult when it was recognized that all activated human T cells transiently express FOXP3. Indeed, although TGF-β and IL-2-stimulated human $CD4^+$ T cells express FOXP3, their TSDR remains methylated (2, 28, 29), a phenotype indicative of cells that are not stably committed to the Treg lineage. In addition, there are controversial findings on whether the resulting cells are

suppressive, with some studies finding suppressive function (30), and others not (2, 28, 29, 31). It is important to note that human Treg suppression assays are particularly difficult to interpret when *in vitro* cultured cells are used due to non-specific effects mediated by media consumption and cell killing (32). Therefore, analysis of the TSDR status, and not functional assays, may be a more reliable way to measure human iTreg development. Collectively, these data suggest that while TGF-β may be necessary for differentiation of mouse and human pTregs *in vivo*, it is likely not sufficient, with other unknown environmental factors needed for their full development.

Interestingly, activated human Tregs express high levels of latent TGF-β coupled to latency-associated peptide and bound to the cell surface protein GARP (33–35). Therefore, Tregs themselves can drive the generation of new pTregs by providing a source of TGF-β (36, 37), offering a molecular explanation for a process termed "infectious tolerance" that has been observed for many years in animal models of transplantation (38–40). Mucosal DCs are also a rich source of TGF-β because they express integrin $\alpha_v\beta_8$, which converts extracellular latent TGF-β to its active form (41, 42). These cells may therefore be particularly important for the differentiation of intestinal pTregs that, as discussed in more detail below, are required for intestinal homeostasis.

Based on evidence that in humans TGF-β alone does not induce robust differentiation of stable Tregs, many studies have sought to define whether addition of other cytokines and/or compounds can enhance the effect (43). The most convincing evidence comes from addition of either the vitamin A metabolite all trans retinoic acid (ATRA, discussed further in the Section "Metabolites" below) or the mTOR inhibitor rapamycin. In mice, ATRA can be effectively generated by mucosal DCs and functions to enhance TGF-β-mediated pTreg generation, (44–46). In humans, suppressive iTregs can be generated with ATRA and TGF-β, but their stability based on the methylation of the TSDR is unknown (47–49). Similarly, addition of rapamycin enhances TGF-β-induced FOXP3 expression (49), and although the stability of these cells is unknown, there is an ongoing clinical trial to test their potential as a cellular therapy in hematopoietic stem cell transplantation (NCT01634217). Notably, rapamycin can also increase the stability of fully differentiated human Tregs *in vitro* (29), and of adoptively transferred non-human primate Tregs *in vivo* (50). These data provide a strong rationale to consider using rapamycin therapy to promote Treg function *in vivo*.

THE ROLE OF CYTOKINES IN Treg HOMEOSTASIS

After development, naïve and memory Tregs in both mice and humans continue to rely heavily on IL-2 signaling for survival and homeostasis. IL-2 may also be important for facilitating Treg survival because it upregulates expression of pro-survival protein myeloid leukemia cell differentiation 1 (MCL1), which counter-regulates the FOXP3-induced pro-apoptotic protein BCL-2-interacting mediator of cell death (BIM) (51, 52). Indeed, administration of IL-2 to mice enhances Treg survival *in vivo* and reduces expression of the pro-apoptotic protein caspase 3 (53). In mice and humans, IL-2 also maintains Treg function by inducing FOXP3 mRNA, stabilizing FOXP3 protein expression, and regulating key Treg-signature molecules such as CTLA-4 and glucocorticoid-induced tumor necrosis factor receptor related protein (GITR) (11).

IL-2 is also essential to prevent the polarization of Tregs into pro-inflammatory effector cells (54, 55). For example, IL-2 signaling in Tregs is required to sustain expression of the GATA-binding protein 3 (GATA3) transcription factor (55, 56). Although this protein is commonly thought of as a Th2 cell lineage-defining protein, its expression is required for negative regulation of the *TBX21* and *RORC* loci, which encode two transcription factors that feedback to diminish FOXP3 expression (55). It is currently not clear whether the role of GATA3 in Tregs is due to direct binding of GATA3 to regulatory regions in the *TBX21* and *RORC* loci, or indirect via positive regulation of FOXP3 itself, which can then repress *TBX21* and *RORC* transcription.

Whether or not other cytokines that signal via γ_c can substitute for IL-2 during pTreg development/survival *in vivo* remains unclear. Of note, some murine memory Tregs residing in the skin, or accumulating with age seem to preferentially rely on IL-7 or IL-15 for homeostasis (57, 58). Although human Tregs can definitely proliferate in response to IL-15 (59, 60), the relevance of IL-7 in humans is unclear as the lack of IL7Rα expression is a defining feature of human Tregs (4, 5).

Because of the essential role of exogenous IL-2 for keeping Tregs alive and maintaining FOXP3 expression, therapeutic approaches that deliver IL-2 signals specifically to Tregs are being actively explored. For example, delivery of IL-2/anti-IL-2-antibody complexes in pre-clinical studies stimulates Treg expansion and reduces disease in models of type 1 diabetes (T1D), experimentally induced autoimmune encephalomyelitis (EAE), collagen-induced arthritis, and angiotensin II-induced aortic stiffening (61–64). Similarly, in models of proteinuric kidney disease and renal ischemia-reperfusion injury, administration of IL-2/anti-IL-2-antibody complexes promotes Treg expansion, improves renal function, and reduces inflammation and disease symptoms (65, 66). In clinical trials, low-dose IL-2 therapy has been investigated for the treatment of graft versus host disease (GVHD) and T1D and appears to successfully expand the circulating Treg cell pool (67–70). A major caveat, however, is finding a dose regimen of IL-2 that only affects Tregs and does not activate $CD8^+$ T cells and NK cells in parallel, as recently observed in a clinical trial of low dose IL-2 and rapamycin in T1D (71, 72). Another consideration is that IL-2-based therapies might not work in subjects who have genetic defects in IL-2R-signaling such as patients with a T1D-susceptibility IL-2RA haplotype (73) or whose Tregs have become IL-2-unresponsive (74).

A converse application of IL-2 targeted therapy is blockade of IL-2, which could theoretically be beneficial in the setting of cancer where depletion of Tregs could boost anti-tumor immunity (75). Interestingly anti-CD25 mAbs (basiliximab, daclizumab) were originally developed as immunosuppressive agents designed to deplete effector T cells and are still in common use today in transplantation. Investigation into whether daclizumab may also affect Tregs has revealed that it does indeed cause a reduction in Tregs by approximately 50%, both in the setting of autoimmunity (multiple sclerosis) and in cancer immunotherapy (76, 77). Basiliximab has similar effects in transplantation (78). However, post daclizumab therapy, the remaining 50% of Tregs are

fully functional (77). These data suggest that, at least using current agents, IL-2 blockade is actually not a very effective way to deplete Tregs, possibly because of their elevated expression of the high affinity IL-2 receptor (i.e. CD25) and/or the ability of other cytokines to compensate *in vivo*.

Another cytokine that has recently gained interest as a regulator of Treg biology is IL-33, a member of the IL-1 cytokine family that signals via a heterodimeric receptor consisting of interleukin-1 receptor-related protein ST2 and the IL-1 receptor accessory protein IL1RAcP (79). Expressed by stromal and immune cells, IL-33 is well known to have a pathological role in airway inflammation and arthritis because it enhances and prolongs immune activation (80). Surprisingly, however, IL-33 treatment can actually protect against experimental colitis and rejection of HLA-mismatched cardiac allografts in mice by promoting Th2 cells and FOXP3$^+$ Tregs (81–83). This anti-inflammatory effect of IL-33 on Tregs seems to be mediated in part via DCs, as IL-33-dependent expansion of murine ST2$^+$FOXP3$^+$ Tregs requires secretion of IL-2 by ST2$^+$ DCs exposed to IL-33 (84).

In some tissues, however, there may be direct effects of IL-33 on Tregs. For example, more than 50% of colonic Tregs express ST2 enabling them to quickly respond to IL-33 released by epithelial cells upon tissue damage (85). Functionally, IL-33 can increase TGF-β-induced proliferation of colonic ST2$^+$ Tregs *in vitro* and stabilize FOXP3 expression in inflamed tissues *in vivo* (85). Notably, IL-23, which is known to inhibit pTreg differentiation (86, 87), reduces expression of ST2 on Tregs (85), resulting in abrogation of the IL-33-mediated increase in pTreg induction and stabilization. Therefore, the balance between IL-33 and IL-23 may be an important factor in determining the outcome of tissue localized immune responses. In humans, IL-33 was previously thought to be an attractive target for therapeutic blocking (88), as a variety of inflammatory diseases feature elevated serum levels of IL-33. However, in light of its newly discovered function in promoting Treg expansion and function, inhibition of IL-33 could also have deleterious effects in some settings.

CONTROL OF Treg FUNCTION BY CYTOKINES

The function of Tregs is also controlled by local the cytokine milieu, with mounting evidence that the presence of pro-inflammatory cytokines affect Treg suppression both directly and indirectly. Cytokines with direct effects on Tregs, such as tumor necrosis factor alpha (TNF-α), provide possible therapeutic targets for modulating Treg function. TNF-α is a pleiotropic cytokine that can act on a wide range of cells. Tregs express the TNF receptor, and there is evidence for both positive and negative effects of TNF-α on their function. Recent evidence shows that TNF-α induces expression of protein phosphatase 1 (PP1), which dephosphorylates the C-terminal DNA-binding domain of FOXP3, resulting in a reduction in its function as a transcription factor (89). Notably, treatment of rheumatoid arthritis subjects with TNF-α-antibodies restores Treg function, decreases PP1 expression, and increases FOXP3 phosphorylation. These data are consistent with previous studies showing that TNF-α impairs Treg function in rheumatoid arthritis by reducing FOXP3 expression (90), and that Tregs expressing membrane-bound TNF-α are less suppressive than TNF-α negative Tregs (91). TNF-α also impairs TGF-β-induced pTreg development in EAE by reducing FOXP3 transcription (92).

Data reporting negative effects of TNF-α on Tregs contrast to a series of reports showing that TNF-α signaling through the TNF receptor 2, which is expressed by a subset of mouse and human effector and memory Tregs, enhances Treg proliferation and suppressive activity (93, 94). Notably, one of the common side effects of TNF-α therapy is psoriasis (95, 96) and data from mouse models suggest this may be due to an anti-TNF-α-mediated decrease in Treg frequency in the skin (97). Therefore, environmental TNF-α may actually bolster Treg function. Understanding how the local tissues define whether TNF-α has a negative or positive effect on Treg function will be key to understanding the side effects of this very common therapy.

Similar to conventional CD4$^+$ T cells, Tregs respond to lineage-defining cytokines, resulting in differentiation into subsets that seem to mirror classical Th1, Th2, and Th17 cells (98, 99). Th1-like, Th2-like, and Th17-like peripheral CD4$^+$CD45RO$^+$CD127^{low}CD25^{high} memory Tregs can be identified in human peripheral blood on the basis of differential expression of the chemokine receptors CXCR3, CCR4, and CCR6, respectively (100). A major question is whether these subsets of Th-like Tregs are protective or pathogenic. Evidence for the former comes from studies showing that Th-like Tregs remain suppressive and are necessary to provide protection from various diseases (101–114). On the other hand, in humans with autoimmunity and/or inflammation, Th1-like FOXP3$^+$ Tregs that express T-bet, CXCR3 and produce IFN-γ appear to lose their suppressive function (115, 116), and multiple reports have shown that Th17-like Tregs are enriched at inflammatory sites, indicating a potential role in disease pathogenesis (116–120). We have also recently shown the first evidence for IL-13$^+$ Th2-like Tregs, which are significantly increased in the skin, but not the blood, of subjects with systemic sclerosis (121). IL-13 is a pro-fibrotic cytokine that drives tissue fibrosis in this disease and *in vitro* experiments revealed that IL-33 increases the proportion of IL-13-producing Tregs in cultures of skin biopsies from healthy controls. Therefore, in addition to promoting Treg survival as described above, in some cases, IL-33 may cause detrimental changes to Treg function.

METABOLITES

Dietary metabolites are another important environmental factor that influence Treg differentiation and function, especially in the gut. Research on the effect of metabolites on Tregs has particularly focused on vitamins A, D, and tryptophan. Understanding the effect of these and other metabolites on Tregs could identify dietary supplements that enhance Treg-based therapies and novel compounds that enhance *in vitro* expansion of stable Tregs.

VITAMIN A

All trans retinoic acid (ATRA) is the main bioactive metabolite of vitamin A and, as briefly discussed above, is well known to have an important role in the differentiation of pTregs (122, 123). *In vivo*, a major source of ATRA appears to be mucosal DCs which in mice characteristically express CD103 (integrin $\alpha_\epsilon\beta_7$), (44–46, 124–126). Since mucosal DCs also express integrin $\alpha_v\beta_8$, which converts extracellular latent TGF-β to its active form (41),

these cells can drive the synergistic induction of FOXP3$^+$ pTregs, which specifically express gut homing markers, including CCR9 and integrin $\alpha_4\beta_7$ (46). In humans, it has recently been demonstrated that ATRA acts on DCs and gives them the ability to preferentially drive the induction of gut homing Tr1 cells, an IL-10-producing FOXP3$^-$ Treg subset (127). The Tr1 cells produced by ATRA-producing DCs in this study displayed *in vitro* suppressive function, expressed gut homing markers CCR9 and integrin $\alpha_4\beta_7$ and also produced IFN-γ.

As described in the Section "Cytokines," Tregs can convert into Th-like cells in response to different inflammatory cytokines, a phenomenon, which may prove to be an obstacle for their use as immunotherapy. Recent work by Lu et al. has demonstrated that pre-treatment of human CD4$^+$CD25^{high}CD127^{low} Tregs with ATRA almost completely prevents IL-1β/IL-6-driven conversion to Th1/Th17-like cells (128). Upon *in vitro* expansion in the presence of IL-1β and IL-6, ATRA-primed Tregs maintained high FOPX3 expression, suppressive function and were superior to untreated Tregs in preventing xenogeneic GVHD in mice. A similar effect of ATRA has also been observed in a study of individuals with autoimmune hepatitis type 2. Holder et al. demonstrated that the suppressive function of Tregs specific for liver enzyme cytochrome P450IID6 (the main disease autoantigen) was impaired following culture with IL-1β/IL-6; however, this was prevented by the addition of ATRA (129). Therefore, ATRA appears to be important for Tr1 cell differentiation in the gut and for stabilizing Tregs under inflammatory conditions, and has shown potential for therapeutic use in mouse models of colitis and periodontitis (130, 131).

VITAMIN D

Vitamin D metabolites have long been recognized as important immunomodulators and exert their effects by binding to the vitamin D receptor, which is expressed on many immune cells including activated T cells (132). The active vitamin D metabolite calcitriol (1,25-dihydroxy vitamin D_3) can be metabolized from vitamin D in the diet or synthesized in the skin following UV exposure. Calcitriol is known to promote the growth of both FOXP3$^+$ and IL-10 producing Tregs, while inhibiting Th17 cells (133, 134). It has recently been shown that calcitriol also induces expression of skin (CCR10 and CLA) and inflamed tissue (CXCR6) homing receptors in Tregs (135), and that addition of TGF-β enhances calcitriol-driven expansion of FOXP3$^+$ Tregs *in vitro* (136). Furthermore, calcidiol (25-hydroxy vitamin D_3), a vitamin D metabolite similar to calcitriol, has been shown to prime mucosal DCs to induce suppressive IL-10 and IFN-γ producing Tr1 cells (127). A recent study has suggested that calcitriol could be a useful adjunct therapy with allergens in sublingual immunotherapy as it specifically enhanced Treg responses to allergens *in vitro* (137). It is interesting to note that, following treatment with UVB, MS patients had enhanced levels of serum calcitriol, which correlated with increased levels of circulating pTregs (138). This link between UVB exposure, serum levels of vitamin D metabolites, and Treg frequency might contribute to the observed epidemiological associations between environmental UVB exposure and incidence of autoimmune disease (139–141).

METABOLITES THAT ACTIVATE ARYL HYDROCARBON RECEPTORS

Numerous metabolites have been described that can activate the aryl hydrocarbon receptor (AHR), a transcription factor that alters the balance between Tregs and Th17 cells. The direction of this balance shift is though to be ligand-dependent, with some AHR ligands preferentially promoting Tregs and others promoting Th17 cells (142–144). For example, kynurenine, which is produced when tryptophan is catabolized by indoleamine 2,3-dioxygenase (IDO), is an AHR agonist that is important for generating Tregs and inhibiting Th17 cell development (145). Indeed, many tolerogenic cells, such as plasmacytoid DCs (146, 147) produce IDO, and through the production of tryptophan metabolites preferentially induce Tregs. Notably, both IDO and AHR are highly expressed in human placenta, implying that tryptophan metabolites acting via AHR also induces Tregs in pregnancy (148), a process critical for maternal/fetal tolerance (149).

Another tryptophan metabolite, cinnabarinic acid, has been identified as a novel AHR ligand (150) that is also an agonist of the type-4 metabotropic glutamate receptor. Cinnabarinic acid has been shown to prevent onset of EAE in mice following administration of myelin oligodendrocyte glycoprotein peptide through enhancing immune responses that were dominated by Tregs (151). Other dietary metabolites that can act as AHR ligands and promote Tregs include indole-3-carbanole (I3C) and 3,3'-diindolylmethane (DIM), derived from cruciferous vegetables. Treatment of EAE mice with either I3C or DIM completely protects against disease symptoms, significantly reduces immune cell infiltration into the CNS, increases Tregs, and reduces Th17 cells (152). These effects are AHR-dependent as treatment with an AHR antagonist reversed the protective effects of I3C and DIM. Similarly, a study of methionine–choline-deficient (MCD)-diet induced mouse nonalcoholic steatohepatitis (NASH) found that administering DIM reduced disease and shifted the immune dominance from Th17 cells toward Tregs using AHR-dependent mechanisms (153).

Studies of these natural metabolites have also led to the identification of novel AHR ligands, such as benzimidazoisoquinolines, which are not part of a normal diet. Administration of these compounds to mice increased Treg frequency and suppressed GVHD in an AHR-dependent manner (154). Understanding how the activity of AHR controls the balance between Tregs and inflammatory T cells will lead to new approaches to alter this balance therapeutically (155).

PURINE METABOLISM

Another important metabolic process is purine catabolism, which regulates the balance of pro-inflammatory adenosine 5'-triphosphate (ATP) and immunosuppressive adenosine. Through expression of adenosine receptors and the ecto-enzymes CD39 and CD73 that metabolize ATP, Tregs are able to both react to, and modulate, immune purinergic signals. CD39 and CD73 function to sequentially catabolize extracellular ATP: CD39 catalyzes the conversion of ATP into adenosine diphosphate (ADP) and adenosine monophosphate (AMP); and CD73 converts AMP into adenosine (156). Whereas ATP signals through type 2 purinergic (P2) receptors to initiate pro-inflammatory responses, adenosine, signals through type 1 purinergic (P1) receptors to suppress immune responses (157).

Mouse Tregs express both CD39 and CD73 and the production of adenosine by these enzymes is thought to be one of the Treg mechanisms of suppression (158). In contrast, most human $CD39^+$ Tregs do not express CD73, and it is thought that human Tregs primarily generate adenosine when they are in proximity to $CD73^+$ cells (159). Interestingly, expression of CD39 enables DCs and neutrophils to move along an ATP concentration gradient to sites of inflammation (160–162), and this may also be true for Tregs. Evidence using the EAE model showing that CD39 has an important role in directing migration of Tregs to lymphoid draining sites of the central nervous system (163) supports this hypothesis.

Regulatory T cells themselves can also respond to adenosine (157), which signals through the A2 class of P1 receptors to stimulate a positive feedback loop by increasing expression of *CD73* mRNA via stimulation of cyclic AMP (cAMP) response elements in the *CD73* locus (164). Adenosine can also act in an autocrine manner via A2A receptors expressed on Tregs to enhance their generation, CTLA-4 expression, and suppressive function (165).

Interestingly, in comparison to the negligible levels of cAMP levels in conventional T cells, human Tregs generate and maintain high intracellular levels of cAMP (166–168). CD39 may be important in this process, as intracellular production of cAMP is increased by extracellular adenosine signaling through A2 receptors. Of note, Tregs can mediate suppression by transferring cAMP through gap junctions into neighboring conventional T cells and DCs (166, 169). Intracellular cAMP also positively feedbacks on Tregs stimulating upregulation of both CTLA-4 (170) and CD39 expression (171).

In terms of the effects of cAMP on pTreg differentiation, in mice there may actually be negative effects as cAMP can suppress TGF-β-driven differentiation of pTregs *in vitro* (172). This negative effect of cAMP is likely due to cAMP-mediated activation of protein kinase A, which enhances TGF-β-mediated activation of mitogen-activated protein kinases ERK and JNK. In humans, however, the effect of cAMP on pTreg differentiation may be different as studies of prostaglandin E2 and vasoactive intestinal peptide, compounds that increase cAMP levels, result in increased pTreg generation and function (172, 173). As growing evidence shows that signaling through G-protein coupled receptors, which stimulate the cAMP pathway, has major effects on Treg differentiation and function (discussed in Section "Microbiome"), developing a full understanding of how cAMP affects Treg biology will be an important area of future research.

MICROBIOME

There are approximately 1000 species of different microbes colonizing the gut, with densities of 10^4-10^5 bacteria per millimeter of effluent in the proximal small intestine and 10^{11} bacteria per gram of luminal content in the colon (174). The high microbial content in the large intestine poses a large challenge to the mucosal immune system, as it needs to tolerate commensal microbiota and dietary antigens while maintaining the ability to eliminate pathogens. Induction of colonic Tregs is crucial in fostering this immune homeostasis.

It is now appreciated that a major site for development of pTregs is the colon, resulting in a large population of regulatory cells that have a distinct TCR repertoire and are critical for intestinal homeostasis (175). Since colonic pTregs are significantly reduced in germ-free mice, commensal microbiota has an essential role in inducing these cells (175–177). Similarly, the development of pTregs in the liver (178) and lungs (179) also requires the presence of commensal microbiota early in life. The exact mechanism behind the induction of colonic pTregs remains unknown, but several microbial components have been found to enhance their expansion and function, including short-chain fatty acids (SCFAs) (180–182), and the bacterial molecule polysaccharide A (PSA) of *Bacteroides fragilis* (183, 184) (**Figure 1**).

SHORT-CHAIN FATTY ACIDS

Despite substantial individual variation in the composition of the microbial community, in healthy adults most gut bacteria belong to two phyla: Firmicutes and Bacteroidetes (185). Because of the large anaerobic community and low oxygen availability in the colon, bacterial metabolism is dominated by fermentation and anaerobic respiration where nitrate, sulfate, and other compounds are used as electron acceptors (186). Undigested dietary carbohydrates are fermented to produce gases and organic acids, particularly the SCFAs acetate (C2), propionate (C3), and butyrate (C4), typically at a ratio of 3:1:1, respectively (187). Of these three main SCFAs, acetate can be produced by enteric bacteria and acetogens; propionate is a by-product of the succinate pathway in Bacteroidetes; and butyrate is formed from two acetyl-CoA molecules in Firmicutes (188). Specific species that have been recognized by their high levels of butyrate production include *Faecalibacterium prausnitzii* and the cluster IV and XIVa of genus *Clostridium* (189). SCFAs are the most abundant (50–100 mM) in the proximal colon, where most fermentation occurs (190). However, in the peripheral blood, only acetate remains in relatively high concentrations, since butyrate is preferentially utilized by colonocytes as an energy source and propionate is metabolized by hepatocytes (190).

Recent work has revealed that SCFAs are important in promoting the differentiation of colonic Tregs. An early study reported that germ-free mice have reduced colonic Treg numbers, and that colonization by bacterial strains belonging to the cluster IV and XIVa of the genus *Clostridium* rescues the deficiency and protects mice from colitis (176). Administration of acetate, propionate, and butyrate in drinking water mimics the effect of *Clostridium* colonization in germ-free mice, resulting in an elevated Treg frequency in the colonic lamina propria and increased IL-10 production by these Tregs (180, 182).

Of the three main SCFAs, butyrate has been found to be the most potent inducer of colonic Tregs. Mice fed a diet enriched in butyrylated starches have more colonic Tregs than those fed a diet containing propinylated or acetylated starches (181). Arpaia et al. tested an array of SCFAs purified from commensal bacteria and confirmed butyrate was the strongest SCFA-inducer of Tregs *in vitro* (180). Mechanistically, it has been proposed that butyrate, and possibly propionate, promote Tregs through inhibiting histone deacetylase (HDAC), causing increased acetylation of histone H3 in the *Foxp3* CNS1 region, and thereby enhancing FOXP3 expression (180, 181).

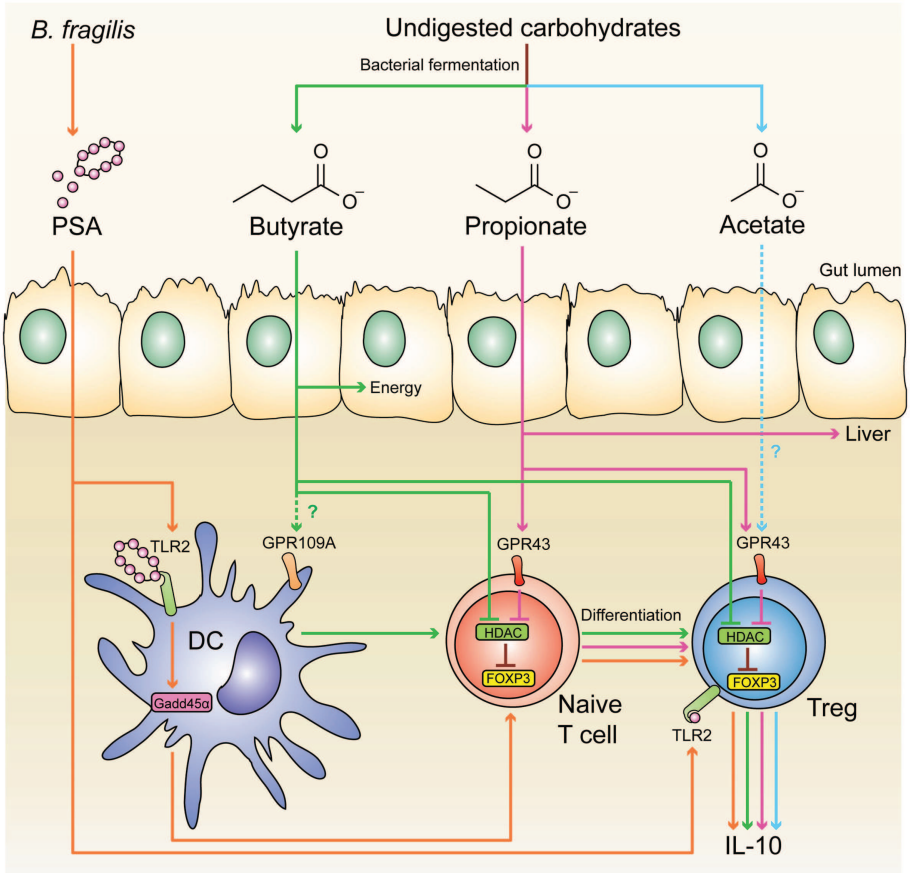

FIGURE 1 | Microbial-derived molecules promote colonic Treg differentiation. Undigested dietary carbohydrates are fermented by gut commensal bacteria to produce the SCFAs acetate, propionate, and butyrate. Administration of acetate in drinking water results in the accumulation of IL-10$^+$ colonic Tregs, and this effect is independent of HDAC inhibition and acetylation of the *Foxp3* CNS1 region. Although acetate is a potent GPR43 ligand, it is not clear whether acetate mediates its effect through this receptor. GPR43 expression in colonic Tregs is required for propionate to inhibit HDAC function and enhance FOXP3 expression, thereby promoting Treg differentiation and IL-10 production, Butyrate has similar effects by either directly acting on Tregs or through modulating DC function to enhance their Treg-inducing ability; however, the role of GPR109A in these effects is controversial. Purified PSA derived from *B. fragilis* can also directly act on Tregs through TLR2 to promote Treg function by enhancing expression of effector molecules including IL-10, TGF-β2, and granzyme B. Membrane-bound PSA cannot act directly on Tregs, instead it interacts with TLR2 on DCs to promote Treg differentiation in a Gadd45α-dependent manner.

Short-chain fatty acids partially mediate their effects through G-protein coupled receptors (GPR), including GPR41, GPR43, and GPR109A. GPR41 and GPR43 are stimulated by all three major SCFAs (191), whereas GPR109A only interacts with butyrate (192). In mice, colonic and small intestinal Tregs express GPR43, and expression of this receptor is required for propionate-mediated HDAC inhibition and Treg expansion (182). There are conflicting results as to whether GPR109A is required for butyrate to mediate its pro-Treg effect. In both mice and humans, GPR109A is only expressed on DCs and macrophages, but not on T cells (192). Singh et al. found that splenic DCs from *Gpr109a*$^{-/-}$ mice were not able to induce Treg differentiation in response to butyrate (192). Additionally, the study found that butyrate treatment increases transcription of *Aldha1* (aldehyde dehydrogenase) in a GPR109A-dependent manner (192). As discussed above, this enzyme is important in vitamin A metabolism, so these data suggest that GPR109A signaling may increase ATRA production by APCs and indirectly promote Treg differentiation and function. In contrast, Arpaia et al. reported that butyrate-pre-treated *Gpr109a*$^{-/-}$ DCs are not defective in *in vitro* generation of Tregs (180). The reason for these discrepant findings is not clear, but overall the emerging data demonstrating that SCFAs can have both direct and indirect effects on Tregs and have opened up an exciting new area of research.

POLYSACCHARIDE A

Another microbial component capable of enhancing Treg function is PSA from the commensal gut bacterial strain *B. fragilis*. The initial study reported that in germ-free mice either colonization by *B. fragilis* or administration of purified PSA induces IL-10 secretion by CD4$^+$ T cells and reduces gut inflammation (193). A subsequent study confirmed that the IL-10-expressing CD4$^+$ T cells were FOXP3$^+$ Tregs and that PSA treatment increases Treg frequency and their expression of effector molecules including IL-10,

TGF-β2, granzyme B, and CCR6 (184). Notably, the authors found that in the absence of APCs, PSA acts directly on Tregs through toll-like receptor 2 (TLR2) to induce the observed effects (194). It has also been demonstrated that administration of PSA protects against induction of EAE in mice through TLR2-mediated expansion of $CD39^+$ Tregs (195).

It remains unknown how PSA is recognized by the mucosal immune system. Since the genome of *B. fragilis* does not contain genes for any known bacterial secretion system (196) and PSA is a large capsular polysaccharide (197), it has been proposed that *B. fragilis* delivers PSA by secreting outer membrane vesicles (OMVs) (198). Shen et al. observed that oral administration of PSA-containing OMVs purified from *B. fragilis* is sufficient to protect mice from experimental colitis and that TLR2 expression on DCs, but not T cells, is required to promote IL-10 production by Tregs (198). Subsequent work identified that PSA-treated plasmacytoid DCs, but not conventional DCs, are responsible for inducing IL-10-secreting Tregs (199). Therefore, while Tregs can directly respond to purified PSA, immune responses to membrane-bound PSA require $TLR2^+$ DCs. It is worth noting that many other TLR ligands can also directly or indirectly impact Treg function [reviewed in Ref. (200)]. In contrast to PSA, other TLR2 ligands, such as Pam3CSK4 and FSL-1, inhibit the function of both mouse and human Tregs (201–204). How multiple TLR signals are integrated in the mucosal environment is unknown.

PROBIOTICS

Long before the direct effects of microbial products on Tregs were understood at the molecular levels, many groups have been exploring the potential therapeutic use of bacteria in the form of probiotics to modulate Tregs. For example, administration of a five-strain probiotic mixture (designated IRT5, including *Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactobacillus reuteri*, *Bifidobacterium bifidum*, and *Streptococcus thermophilus*) in mice increases the proportion of Tregs in the mesenteric lymph nodes (205). $CD11c^+$ DCs purified from these treated mice also had higher expression of IL-10, TGF-β, and IDO, and were more capable of inducing Treg differentiation compared to DCs from control mice (205). A more recent study demonstrated that administration of *L. reuteri* to mice was sufficient to prevent high-fat-diet-induced adipose inflammation and obesity, an effect that was associated with enhanced Treg induction and IL-10 expression (206). *In vitro*, *L. casei* and *L. reuteri* can also prime human monocyte-derived DCs to stimulate IL-10-producing Tregs through the adhesion molecule DC-SIGN (207). Lopez et al. found that DCs exposed to *B. bifidum* membrane vesicles strongly induced Treg differentiation *in vitro*, suggesting that the potential use of the membrane vesicle as a safe adjunct therapy (208).

Although much work is still needed to elucidate the details of how commensal microbiota induce Tregs, numerous randomized trials in the past decade using *Lactobacillus* and *Bifidobacterium* to treat inflammatory disorders have already demonstrated the clinical benefit of this approach (209). Indeed, while delivery of purified PSA or SCFAs may represent an effective, transient therapy, the use of probiotics may offer a well-tolerated long-term therapeutic solution to enhancing intestinal immunoregulatory cells.

CONCLUSION

Environmental stimuli influence all aspects of Treg biology: from development and differentiation to migration and function. As well as refining our understanding of how well-described cytokines affect Tregs, we are also discovering new cytokines, such as IL-33, which have a critical role in Treg function (**Table 1**). Other key factors influencing Tregs, particularly in the gut, are dietary metabolites, catabolites, and bacterial components from the microbiome. There is emerging evidence that retinoic acid is a key metabolite for expanding a stable population of Tregs, data that have clear implications for developing therapeutic approaches. Furthermore, aspects of the microbiome clearly help determine which commensal antigens the immune system is educated against and have a previously unappreciated role in influencing T cell differentiation in the gut. Further studies in this area will expand our knowledge of T cell biology and hopefully uncover new elements of disease pathogenesis and guide the development of Treg-based therapies.

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Vitamin D actions on CD4$^+$ T cells in autoimmune disease

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This review summarizes and integrates research on vitamin D and CD4$^+$ T-lymphocyte biology to develop new mechanistic insights into the molecular etiology of autoimmune disease. A deep understanding of molecular mechanisms relevant to gene–environment interactions is needed to deliver etiology-based autoimmune disease prevention and treatment strategies. Evidence linking sunlight, vitamin D, and the risk of multiple sclerosis and type 1 diabetes is summarized to develop the thesis that vitamin D is the environmental factor that most strongly influences autoimmune disease development. Evidence for CD4$^+$ T-cell involvement in autoimmune disease pathogenesis and for paracrine calcitriol signaling to CD4$^+$ T lymphocytes is summarized to support the thesis that calcitriol is sunlight's main protective signal transducer in autoimmune disease risk. Animal modeling and human mechanistic data are summarized to support the view that vitamin D probably influences thymic negative selection, effector Th1 and Th17 pathogenesis and responsiveness to extrinsic cell death signals, FoxP3$^+$CD4$^+$ T-regulatory cell and CD4$^+$ T-regulatory cell type 1 (Tr1) cell functions, and a Th1–Tr1 switch. The proposed Th1–Tr1 switch appears to bridge two stable, self-reinforcing immune states, pro- and anti-inflammatory, each with a characteristic gene regulatory network. The bi-stable switch would enable T cells to integrate signals from pathogens, hormones, cell–cell interactions, and soluble mediators and respond in a biologically appropriate manner. Finally, unanswered questions and potentially informative future research directions are highlighted to speed delivery of etiology-based strategies to reduce autoimmune disease.

Keywords: vitamin D, CD4-positive T lymphocytes, autoimmune diseases, multiple sclerosis, type 1 diabetes

INTRODUCTION

Autoimmune diseases afflict ~50 million Americans and contribute >$100 billion to US health care costs (1). The global burden has risen with the near tripling in the last half-century of multiple sclerosis (MS) (2, 3), type 1 diabetes (T1D) (4), and other autoimmune diseases. A deep understanding of disease mechanisms will be needed to deliver etiology-based strategies to reverse this vexing trend. Indeed, "functional and mechanistic work on the molecular etiology of disease remains one of the major challenges in modern biology" (5).

This review highlights recent advances in vitamin D and T-lymphocyte biology in an effort to harness vitamin D's potential to reduce the impact of autoimmune diseases. Gene–environment interactions, sunlight and vitamin D, and T lymphocytes as autoimmune disease initiators and vitamin D targets are discussed to explain the rationale for reviewing vitamin D mechanisms in T cells. Research on vitamin D regulation of thymocyte selection, Th1 and Th17 cells, T-cell programed cell death, and T-regulatory (Treg) cells is summarized and integrated into model mechanisms. Finally, unanswered questions relating to vitamin D mechanisms in CD4$^+$ T cells are highlighted to promote further research that may lead to a deeper understanding of autoimmune disease molecular etiology.

Abbreviations: 25-OHD$_3$, 25-hydroxyvitamin D$_3$; APC, antigen-presenting cells; calcitriol, 1α,25-dihydroxyvitamin D$_3$; CNS, central nervous system; CTLA4, cytotoxic T-lymphocyte antigen 4; DC, dendritic cell; EAE, experimental autoimmune encephalomyelitis; EAU, experimental autoimmune uveitis; EBV, Epstein-Barr virus; IFN, interferon; IL, interleukin; MHC, major histocompatibility complex; MRI, magnetic resonance imaging; MS, multiple sclerosis; NOD, non-obese diabetic; RANK, receptor-activator of NF-kappaB; RANKL, receptor-activator of NF-kappaB ligand; T1D, type 1 diabetes; TCR, T-cell receptor; Th, T helper; TLR, Toll-like receptor; Tr1, T-regulatory cell type 1; Treg, T-regulatory; TYK2, tyrosine kinase 2; UV, ultraviolet; VDR, vitamin D receptor; VDRE, vitamin D response element.

GENES, SUNLIGHT, VITAMIN D, AND T LYMPHOCYTES

AUTOIMMUNE DISEASES

Autoimmune diseases represent a failure of self-identification leading to an immune-mediated assault on host tissues. More than 100 autoimmune syndromes exist (1). We drew mainly on MS and T1D research because intensive investigation has generated detailed insights into vitamin D mechanisms in these diseases and provided valuable guidance for research on other autoimmune diseases. Other autoimmune diseases are included where robust mechanistic data exist. A recent chapter (6) and a review (7) have summarized vitamin D mechanisms in autoimmune diseases more generally.

Multiple sclerosis and T1D have distinct target organs, genetic risk factors, onset ages, and female to male ratios, but target

organ-specific T cells as initiators unite these diseases. MS is the leading cause of non-traumatic neurological disability in young adults. It results from an autoimmune attack on the axon–myelin unit (8). Neurological dysfunction in MS is attributed to focal demyelinated lesions in the central nervous system (CNS). The neurodegenerative process is believed to occur sub-clinically before the disease is typically diagnosed in the third decade of life. Most MS patients have a relapsing-remitting form of the disease, and among them, women outnumber men 3:1.

Type 1 diabetes is a common chronic disease of childhood with an onset typically between ages 6 and 15. Boys with T1D slightly outnumber girls. T1D results from an autoimmune attack on insulin-producing pancreatic β-cells (9). Metabolic dysfunction in T1D is attributed to β-cell destruction and loss of insulin production and blood glucose control. The β-cell degenerative process is also believed to occur sub-clinically before a T1D diagnosis, when as much as 70% of β-cell mass has been destroyed and insulin supply no longer meets demand (10). We searched for analogies between MS and T1D that might reveal over-arching environmental influences on T lymphocytes in autoimmunity.

GENE–ENVIRONMENT INTERACTIONS IN AUTOIMMUNE DISEASE

Interacting genetic, environmental, and hormonal influences drive autoimmune disease. There is a weak genetic component in autoimmune disease susceptibility. MS risk increases a modest 2–6% among first-degree biological relatives of an MS index case (11), but candidate risk loci are of such modest aggregate influence that genetic burden scores cannot accurately predict disease (12, 13). Autoimmune disease discordance between monozygotic twins is generally between 60 and 75% and cannot be explained by somatic mosaicism in MS (14), T1D (15), Crohn's disease (CD) (16, 17), or systemic lupus erythematosus (SLE) (18). The low penetrance of candidate risk loci, high monozygotic twin discordance rates, and rapidly rising disease incidence rates underscore the hypothesis that *an autoimmune disease phenotype emerges when modifiable environmental stressors act on a disease-susceptible genotype, and exposure to at least one environmental stressor is increasing.* The key questions are (i) what are the dominant, modifiable environmental stressors, (ii) by what mechanisms do they interact with susceptibility genes to propel the disease process, and (iii) can mechanistic knowledge of gene–environment interactions be exploited to craft effective, etiology-based disease prevention and treatment strategies.

SUNLIGHT EXPOSURE AND RISK OF MS AND T1D DISEASE

There is a large, latitude-linked, non-transmissible environmental component acting in a female-biased manner at the population level that determines whether the MS phenotype emerges from an MS-susceptible genotype (19, 20). Supporting this interpretation are the gradient in MS prevalence as a function of latitude (21, 22), alteration of MS risk by migration (23), and the equivalent MS risk between non-biological relatives of an MS case and the general population (24). The latitudinal gradient has dissipated as incidence has risen in low latitude regions. Peak MS prevalence was ~$130/10^5$ population in 1960 (21) and is now ~$400/10^5$ (22). Young women have borne the brunt of rising MS incidence (25, 26). The rise in female MS incidence implies a significant proportion of new female MS cases may be preventable (27).

Low sunlight exposure is postulated to be the major latitude-linked component in MS risk (21). Ultraviolet (UV) irradiance varies 400-fold with latitude (28), correlating inversely with the ~400-fold variation in MS prevalence (22). UV irradiance also varies seasonally at high latitudes, where increases in MS disease activity have been noted lagging seasonal declines in ambient UV light (29–31). Furthermore, childhood and occupational sunlight exposure correlated inversely with MS disease risk (32–35) and mortality (36).

Low sunlight exposure is also postulated to be a major component in T1D risk. Globally, T1D incidence varied ~350-fold (37) correlating inversely with the 400-fold latitudinal variation in UV irradiance (28). Global T1D incidence is increasing, while the proportion of T1D patients with the major *HLA* risk genotype is decreasing (13), implicating a modifiable environmental factor. T1D onset peaked between October and January and reached a nadir between June and August in the northern hemisphere, with a reverse pattern in the southern hemisphere (38). This correlation disappeared after adjustment for latitude. The inverse correlation between ambient winter UV radiation and T1D ($r = -0.80$) (39) was nearly as robust as that for MS ($r = -0.9$) (21). Thus, ambient UV irradiance is the leading candidate for the large, latitude-linked environmental risk factor in both MS and T1D (40, 41).

The correlative data are consistent with but do not prove that low sunlight exposure is a major component in autoimmune disease risk. Other environmental risk factors have been proposed (42), but they have markedly smaller effect sizes. For example, symptomatic Epstein-Barr virus (EBV) infection in adolescence (mononucleosis), the strongest of the environmental risk factors that appear to act independently of latitude, season, and UV light (43), correlates with a two to threefold increase in MS risk (44). In sharp contrast, residence in a low ambient UV light region correlates with a >100-fold increase in MS risk (22). Whether EBV infection and UV light are completely independent risk factors is currently debated (45). As discussed below, genetic data suggest the *VDR* gene influences HLA-DRB1 presentation of peptides to $CD4^+$ T lymphocytes, and structural data show pathogenic T cells did not distinguish a *DRB1**1501-restricted myelin peptide from a *DRB5**0101-restricted EBV peptide suggesting a molecular mimicry mechanism underlying pathogenesis. In summary, the UV-linked component exerts its influence on multiple autoimmune diseases and on populations with disparate ancestries, distinct dietary and smoking habits, and dissimilar exposures to infectious and commensal organisms. These qualities of strength and universality provide the rationale for close investigation of this component's identity and mechanisms.

VITAMIN D AND TRANSDUCTION OF SUNLIGHT'S PROTECTIVE SIGNALS IN MS AND T1D

Vitamin D_3 and its hormonal form, 1α,25-dihydroxyvitamin D_3 (calcitriol) have been proposed as the major biological transducers of sunlight's protective signals in autoimmune disease due to calcitriol's ability to selectively regulate T-cell-mediated autoimmune responses (46). Evidence supporting this hypothesis has been reviewed (6, 7, 42, 47, 48). Persuasive evidence for vitamin D

as sunlight's signal transducer derives from genetic linkage studies. Rare loss-of-function mutations in the *CYP27B1* gene correlated with a significantly increased autoimmune disease risk. This association was first reported for T1D (49–54), Addison's disease (55), Hashimoto's thyroiditis, and Graves' disease (56). It was subsequently reported for MS (57–60). In rare multi-incident MS families, 35 of 35 cases inherited one defective *CYP27B1* allele, an inheritance pattern with small odds (one in a billion) of occurring by chance (58). Because *CYP27B1* mutations are highly penetrant but exceedingly rare, they do not contribute genetic risk in the vast majority of disease cases. In fact, genome-wide association studies (GWAS) and some case–control studies did not detect an association between *CYP27B1* variants and MS or T1D (61–65). However, the replicated positive genetic findings indelibly mark calcitriol synthesis as a key determinant of MS and T1D risk.

Correlations between *VDR* alleles and MS susceptibility have also been reported (66–68). An early study found a *VDR* and MS association in patients who carried the high-risk *HLA-DRB1**1501 allele (69). Later research identified a putative vitamin D-responsive element (VDRE; see below) in the *HLA-DRB1**1501 promoter (70). The *VDR* association data have been inconsistent between populations, and some *VDR* polymorphisms studied do not have known functional impacts. The *VDR Fok*I polymorphism is an exception; VDR^{F} (without the *Fok*I site) encodes a 424 amino acid protein with higher transcriptional activity than the 427 amino acid protein encoded by VDR^{f} (a *Fok*I site in the first ATG codon) (71, 72). The less active VDR^{f} protein was associated with higher serum 25-hydroxyvitamin D (25-OHD) levels, lower MS risk, and lower MS disability (73–76). Another exception is the $Cdx\text{-}2^{G}$ variant, which has 70% reduced promoter activity (77); this variant correlated with an increased risk of MS in children who had $\leq$2 h/day of winter sun exposure (78). GWAS did not detect a *VDR* and MS association (61).

Some family studies have also detected linkage between *VDR* polymorphisms and T1D, but concerns about inconsistencies between populations and unknown functional impacts also apply here (79). Reasoning that a *VDR* and T1D association might only be evident if 25-OHD is sufficient to support calcitriol synthesis in cells relevant to T1D, investigators searched for this association as a function of latitude (79). They found a VDR^{F} and T1D association that varied in strength according to ambient winter UV light. These data emphasize the importance of analyzing genetic data in the context of environmental variables. GWAS did not detect a *VDR* and T1D association (62, 80).

Intriguing data suggest an epistatic interaction between *VDR* alleles and *HLA-DR* susceptibility loci in T1D as in MS. The VDR^{F}–T1D association was only evident in patients who carried the high-risk *HLA-DRB1**0301 allele (81). The *HLA-DRB1**0301 allele like the *HLA-DRB1**1501 allele harbors a putative VDRE in its promoter (70). Alleles without the putative VDRE were associated with disease resistance. These parallels suggest an influence of UV light and vitamin D on *HLA-DRB1* gene expression and presentation to $CD4^{+}$ T lymphocytes of peptides relevant to T1D and MS etiology. The nature of the peptides and the timing and outcome of the presentation event are unknown, but could relate to thymic tolerance or peripheral T-cell responses to peptides from infectious agents. In any case, the positive findings regarding *VDR* polymorphisms provide genetic support for calcitriol and vitamin D receptor (VDR)-regulated transcriptional events as determinants of MS and T1D risk.

Additional evidence for vitamin D and calcitriol as sunlight's signal transducers derives from vitamin D studies. An early study closely correlated childhood dental disease, serving as an accessible biomarker of exposure to low vitamin D status (82), with worldwide MS mortality ($r = 0.78$, $p < 0.002$) (83). The first vitamin D_3 interventional study to inhibit autoimmune disease was performed in the murine experimental autoimmune encephalomyelitis (EAE) model of MS (84). Human case–control studies have correlated low vitamin D intake and low circulating 25-OHD with high MS risk (85–87). Importantly, MS risk correlated inversely with circulating 25-OHD independently of personal UV light exposure (88). Abundant data have now correlated circulating 25-OHD inversely with MS disease activity (30, 88–94). These data and the *CYP27B1* and *VDR* evidence contradict the view that UV light's protective effects in demyelinating disease do not involve vitamin D (95).

In MS patients who had low vitamin D_3 levels and were not taking disease-modifying drugs, supplementary vitamin D_3 as a stand-alone intervention significantly reduced disease progression (96), and decreased new lesion formation and progression from optic neuritis to clinically definite MS (97). A vitamin D_2 supplementation study did not report similar findings (98), but significant methodological flaws were noted in that study (99). Moreover, it is well known that vitamin D_2 and vitamin D_3 are not biologically comparable as regards their metabolism and ability to transcriptionally activate the VDR (100–102). Ongoing clinical studies are testing whether vitamin D_3 as an add-on to disease-modifying drug therapy will improve drug efficacy (103). While this is a valid pharmaceutical question, it is separate from the question of whether vitamin D_3 could be an etiology-based intervention to reduce the impact of autoimmune disease. Studies of vitamin D_3 as a stand-alone intervention in individuals who have low vitamin D_3 levels and who are not using disease-modifying drugs will be needed to address the etiological question. Such studies are needed and fully justified.

The T1D data also support vitamin D as sunlight's protective signal transducer (6, 104, 105). The EURODIAB study correlated vitamin D_3 supplementation in infancy with decreased T1D risk (odds ratio 0.67) (106), a finding that has been replicated (107). Finland, at 60–70°N, has the highest T1D incidence in the world (108). Finnish T1D incidence quadrupled as recommended vitamin D_3 intakes for children decreased from 4000 to 400 IU/day between 1965 and 2005 (109). Another retrospective analysis found childhood vitamin D_3 supplementation correlated with an 88% lower risk of T1D (110). These data imply that recommending higher vitamin D_3 intakes for pregnant women, infants, and children might decrease T1D incidence by >75%. A very recent study demonstrated that life-long, high-dose vitamin D_3 supplementation significantly reduced spontaneous diabetes in non-obese diabetic (NOD) mice (111). Finally, vitamin D_3 interventions have improved glycemic control in T1D patients (112–114).

The association of UV irradiance and vitamin D with autoimmune disease risk is strong and consistent, shows a dose–response relationship, is temporally plausible and appears to be universal with respect to genotypes, dietary and smoking habits, and

exposure to infectious and commensal organisms. Thus, it is reasonable to suggest that *vitamin D is probably the environmental factor with the greatest influence on the emergence of an autoimmune disease phenotype given a disease-susceptible genotype*. What remains to be done to satisfy the Bradford Hill criteria (115) is to rigorously test this relationship experimentally in humans, and to uncover plausible biological mechanisms that cohere with known facts of autoimmune disease. There is a growing consensus that a vitamin D_3 interventional study for autoimmune disease prevention is needed and fully justified.

T LYMPHOCYTES IN AUTOIMMUNE DISEASE

The effort to decipher protective vitamin D mechanisms in autoimmunity focuses on T lymphocytes, because in failing to correctly discriminate between self and non-self, auto-reactive T cells drive target organ destruction (116). In animal models, auto-reactive T-cell transfer drives target organ destruction (117). The role of pathogenic auto-reactive T cells as autoimmune disease initiators unites these diseases under a common mechanistic umbrella.

Unequivocal evidence of T-lymphocyte involvement in human T1D came from transplantation studies. Transplanting pancreatic tissue from a healthy subject into his/her T1D-affected identical twin failed as a T1D therapy due to a T-cell-mediated attack on the transplanted pancreatic tissue unless T-cell immunosuppressive therapy was administered (118). Evidence for pathogenic $CD4^+$ T lymphocytes in T1D is now very strong (10, 119).

Original evidence of T-lymphocyte involvement in MS pathogenesis came from studies showing T cells rapidly migrated from the periphery into the CNS of MS patients, where clonally restricted, activated T cells accumulated in nascent MS lesions (120). The T cells from individual MS patients showed dominant usage of specific T-cell receptor (TCR) alpha and beta chains and VDJ sequences, and specificity for myelin basic protein (MBP) peptides presented by HLA class II molecules (121). These observations argue strongly for involvement of HLA class II-restricted, auto-reactive $CD4^+$ T cells in MS pathogenesis (122).

Newer experimental approaches have confirmed the pioneering studies. Analysis of the mRNA transcriptome in blood cells from MS patients pointed to antigen presentation, the immune synapse, and T-cell deregulation in MS pathogenesis (123, 124). GWAS identified genes related to $CD4^+$ T-cell function in regions harboring putative susceptibility loci (61). Analysis of expression quantitative trait loci in autoimmune disease states (125) and genetic analysis of inter-individual variability in T helper (Th) activation as a function of ancestry and autoimmune disease susceptibility (126) also implicated $CD4^+$ Th cell activation in autoimmune disease pathogenesis. In summary, consideration of gene–environment interactions, sunlight and vitamin D, and T lymphocytes as autoimmune disease initiators provides the rationale for investigating vitamin D mechanisms in $CD4^+$ T cells in an effort to understand the molecular etiology of autoimmune disease.

VITAMIN D METABOLISM FROM A T-LYMPHOCYTE PERSPECTIVE

CALCITRIOL'S NON-CALCEMIC ACTIONS

The vitamin D system is an evolutionarily ancient and versatile system that coordinates a plethora of biological processes like cellular metabolism and growth, differentiation and death, organismal growth, reproduction, and immunity according to sunlight's cues. The system's signal transducing molecules are calcitriol, a small lipophilic hormone, and the VDR, a hormone-responsive transcriptional regulator (127). Calcitriol and mammalian VDR orthologs have functioned as sunlight sensors throughout >750 million years of evolution (128, 129). Many of the ancient organisms lacked calcified structures, so the vitamin D system must have originally supported non-calcemic functions (129). We focus on calcitriol's actions in $CD4^+$ T lymphocytes, but actions in other tissues, for example promotion of remyelination in the brain (130), insulin release from the pancreas (131), and intestinal barrier function in the colon (132), undoubtedly contribute to calcitriol's potency as a chronic autoimmune and neurodegenerative disease inhibitor.

ENZYMES OF VITAMIN D ACTIVATION

Vitamin D metabolism has been well described elsewhere (127, 133–136). Cutaneous vitamin D_3 generated by high energy UVB photons (290–315 nm) provides 90% of the human vitamin D requirement (108). Vitamin D_3 synthesis varies seasonally at high latitudes, reaching a nadir 2 months after the winter solstice, and a zenith 2 months after the summer solstice (137). The biological half-life of 25-OHD_3 is ~2 months, so this metabolite effectively integrates sunlight's energy signal over time.

AT HIGH LATITUDES, THERE IS A PERIOD OF "LIGHT STARVATION" WHEN VITAMIN D SYNTHESIS IS NEGLIGIBLE

The higher the latitude, the greater is the period of light starvation. At 42°N, cutaneous vitamin D synthesis is negligible from November through February (138, 139). Vitamin D synthesis decreases with increasing skin pigmentation, sunscreen use, and advancing age. Vitamin D deficiency has rapidly become a worldwide health problem, due to life-style changes (indoor living and working; sunscreen use) that have reduced sunlight exposure (140, 141). Now, more than 75% of Caucasians and >90% of Blacks, Hispanics, and Asians in the USA have 25-OHD_3 <75 nmol/L (30 ng/mL), double the number one decade ago (142). The rapid rise in vitamin D insufficiency correlates temporally with the rapid rise in MS and T1D incidence.

Two hydroxylation reactions produce the calcitriol from vitamin D_3. The *CYP2R1*-encoded 25-hydroxylase converts vitamin D_3 into 25-OHD_3. The *CYP27B1*-encoded 1α-hydroxylase converts 25-OHD_3 into calcitriol. Calcitriol induces *CYP24A1* gene encoding the 24-hydroxylase to convert calcitriol into inactive calcitroic acid. These hydroxylases are cytochrome P450 enzymes (133, 134). They are encoded in nuclear DNA, but the enzymes themselves localize to the mitochondrial membrane.

Many chronic diseases provisionally associated with vitamin D disproportionately impact individuals of African ancestry (AA). Lower serum 25-OHD in people of AA may be contributing to these health disparities (143). Serum 25-OHD levels are subject to genetic regulation (74), and polymorphisms in the *GC*, *DHCR7*, and *CYP2R1* genes have been correlated with circulating 25-OHD_3 levels in people of European ancestry (EA) (144, 145). Motivated by the need to understand racial health disparities, recent research has investigated potential genetic contributions to circulating 25-OHD_3 in AA and EA subjects. The AA subjects had different

GC alleles encoding the vitamin D-binding protein (DBP) and lower circulating 25-OHD (146). In addition to binding vitamin D metabolites, the DBP functions in fatty acid transport, macrophage activation, and chemotaxis (147). A study of older male subjects from urban areas confirmed the association of *CYP2R1* variants with serum 25-OHD after correction for vitamin D intake, season of sampling, BMI, and other variables (148). This association was more robust in EA than AA subjects. The *GC* variants were associated with serum 25-OHD only in the EA subjects. Age, ancestry, vitamin D intake, and season of sampling explained 19 and 24% of the variance in AA and EA subjects, respectively. Adding genetic variants to the model explained an additional 1% (AA) and 4% (EA) of the variance. However, 72% (EA) and 80% (AA) of the variance was unexplained. Thus, genetic variants are very minor contributors to racial disparities in serum 25-OHD. The data also suggest some relevant parameters were either not considered or were subject to a large error in measurement. Unlike other reports, skin pigmentation was unrelated to serum 25-OHD in this study, and UVR exposure was marginally related to serum 25-OHD only in EA subjects. These data indicate serum 25-OHD derived primarily from vitamin D ingestion, which was assessed by a questionnaire. The questionnaire may have introduced error. Whether it assessed vitamin D supplement use was not stated. The authors previously reported that their EA subjects were more likely to use vitamin D supplements than the AA subjects (149). To generate insight into disparities in chronic diseases provisionally associated with vitamin D, it will be essential to discover dominant variables by integrating data on age, gender, life-style choices, skin color, UVR exposure, diet, vitamin D supplement use, and genetic variation with data on 25-OHD and disease phenotype.

PARACRINE CALCITRIOL SIGNALING TO T CELLS

An important question from a $CD4^+$ T-lymphocyte perspective is whether the calcitriol signal derives from the kidney (endocrine signaling) or from tissue-resident cells at sites of inflammation (paracrine signaling). Calcitriol has been classically viewed as an endocrine hormone, with renal synthesis and systemic distribution regulating mineral ion homeostasis and skeletal maintenance in target tissues. Blood calcitriol levels are maintained within very narrow limits and show minimal seasonal fluctuation (150), whereas many autoimmune diseases show fluctuating periods of relapse and remission. For example, MS disease activity fluctuates seasonally, correlating with and lagging changes in ambient UVB sunlight (31) and 25-OHD_3 (29). These data are not consistent with endocrine signaling and instead support paracrine signaling to T lymphocytes.

Evidence that tissue-resident antigen-presenting cells (APC) produce calcitriol (151) and T cells express the VDR (152, 153) first suggested paracrine signaling between immune cells. Adding 25-OHD_3 to cultures altered human T-cell responses only when APC were present to produce calcitriol (154). The APC required activation through pathogen-associated pattern recognition receptors, for example the toll-like receptors (TLR), and/or stimulation by cytokines, most notably interferon-gamma (IFN-γ), interleukin-2 (IL-2), and IL-15, to become calcitriol producers (136, 154–157).

Calcitriol synthesis has now been shown in many non-calcified mammalian tissues frequented by roving T lymphocytes, most notably the skin (158, 159), lung (160), colon (161, 162), brain (163), placenta, and other reproductive tissues (154, 157, 164), so paracrine signaling to T cells is a well-established concept. The *CYP27B1* transcripts are more abundant in tissues with barrier (skin, lung, colon) or reproductive function (maternal decidua, fetal trophoblast, testis) than in the kidney (158), supporting the thesis that biological protection at host–environment interfaces and environmental impacts on reproduction may have driven vitamin D system evolution. Available data suggest that *paracrine calcitriol signaling to T cells within tissues is likely the major pathway by which sunlight exerts its influence on the emergence of an autoimmune disease phenotype.*

It is valuable to consider how calcitriol signaling to $CD4^+$ T lymphocytes could be compromised causing immune-mediated tissue damage. Research in the EAE model of MS demonstrated that insufficient vitamin D_3 disrupted calcitriol synthesis in the CNS (84). Research in the NOD model of T1D demonstrated that reduced 1α-hydroxylase activity in APC disrupted signaling and contributed to diabetes (165). As detailed above, *CYP27B1* gene lesions compromise paracrine signaling. It is not widely appreciated that corticosteroids like prednisone (166), prednisolone (167), or dexamethasone (151) compromise paracrine signaling to $CD4^+$ T cells because they inhibit calcitriol synthesis by activated innate immune cells *in vivo*. Corticosteroid inhibition of calcitriol synthesis in the airway may have contributed to negative results in the VIDA trial of vitamin D_3 supplementation in asthma patients who were receiving concurrent corticosteroid therapy (168).

Deregulation of the *CYP24A1* gene promoter might also disrupt calcitriol signaling and promote a pro-inflammatory state. Epigenetic silencing of the *CYP24A1* promoter in placental tissue promoted calcitriol accumulation and an immune tolerant state at the maternal–fetal interface (169, 170). Expression of the *CYP24A1* gene differed between males and females. In the CNS of rodents with EAE, *Cyp24a1* gene expression was higher and calcitriol responsiveness lower in males than females (84, 171). Similarly in human T cells from MS patients and healthy controls, *CYP24A1* gene expression was higher and calcitriol responsiveness was lower in males than females (172). The rodent and human data suggest males may produce calcitriol at a rate equal to females but inactivate it faster. Gender differences in *CYP24A1* expression and vitamin D metabolism may underlie the stronger inverse correlation between ambient UV light and MS risk in women compared to men (173). Estradiol addition to male T cells decreased the *CYP24A1* transcripts and increased calcitriol responsiveness (172), suggesting estradiol may silence the *CYP24A1* gene to promote calcitriol accumulation (169, 170).

VITAMIN D RECEPTOR EXPRESSION BY $CD4^+$ T LYMPHOCYTES

VITAMIN D RECEPTOR AND VITAMIN D-RESPONSIVE ELEMENTS

The VDR enables cells to respond to calcitriol. Early researchers demonstrated a protein with high affinity (K_d 0.1 nM) for calcitriol in activated human $CD4^+$ T lymphocytes that was later identified as the VDR (152, 153). The VDR is a nuclear protein that dimerizes with the retinoid X receptor to regulate gene expression through VDRE in calcitriol-responsive genes (127). A VDRE is composed of two hexameric half-sites, arranged as

direct repeats separated by three random base pairs, for example *GGTTCA*CGA*GGTTCA* (174, 175). Depending on the type of cell, the ligand-activated VDR–RXR complex recruits either coactivator complexes and cooperating transcriptional machinery or corepressor complexes to determine the nature of the transcriptional response from VDRE-containing target genes.

Antibody specificity problems have confounded investigations of VDR protein expression in T cells. All but one of the commercially available antibodies to the VDR bound non-specifically to cells and tissues from DeMay VDR-null mice (176, 177). These were confirmed by flow cytometry; all available antibodies either stained $CD4^+$ T cells from DeMay VDR-null mice or did not yield a signal (171). The specificity problems have slowed progress in assessing VDR protein expression in T-cell subsets.

VDR AND CD4+ T-CELL ACTIVATION

Resting and activated T cells differ significantly in VDR expression; resting cells expressed fewer than 1000 VDR/cell and activation increased this number 10-fold (178). VDR expression peaked at 48 h post stimulation (179). In rodents, activated $CD8^+$ T cells had higher VDR expression than $CD4^+$ T cells (180).

Vitamin D receptor expression was needed for optimal human T-cell activation *in vitro* (181). Naïve T cells were VDR negative and responded weakly to TCR stimulation. Weak TCR signaling via the mitogen-activated protein kinase p38 pathway induced VDR expression. The T cells subsequently up-regulated phospholipase C-gamma1 (PLC-γ1) expression 75-fold, enabling them to flux calcium and become fully activated. PLC-γ1 induction appeared to be VDR-dependent. The dependence of T-cell activation on VDR expression has not been demonstrated *in vivo*.

VDR AND CD4+ T-CELL SUBSETS

Among rodent $CD4^+$ T-cell subsets, IL-4-producing Th2 cells, IFN-γ-producing Th1 cells, and IL-17-producing Th17 cells all had abundant *Vdr* transcripts (182–185). Importantly, a source of estradiol in female mice (186), and a functional *Ifng* gene in male and female mice (171) were essential for Th1 and Th17 cell *Vdr* gene expression. Recent data on human Th1 and Th17 cells produced *in vitro* also show high *Vdr* gene expression (179).

Whether $CD4^+Foxp3^+$ Treg cells express the VDR is unclear. Very low *Vdr* transcript levels were observed in rodent $CD4^+Foxp3^+$ Treg cells produced *in vitro* (187) and rodent $CD4^+EGFP^+$ Treg cells generated *in vivo* and flow-sorted from the spleens of $Foxp3^{EGFP}$ reporter mice (184). Those *in vivo* studies revealed an inverse relationship between *Foxp3* and *Vdr* gene expression, with many *Foxp3* transcripts and few *Vdr* transcripts in the $Foxp3^+EGFP^+$ T cells and the reverse pattern in the $EGFP^-$ T cells. Consistent with low *Vdr* gene expression, calcitriol had no impact on flow-sorted $CD4^+Foxp3^+EGFP^+$ T cells during activation *in vitro* (184). Calcitriol also had no impact on $CD4^+Foxp3^+$ T-cell proportions in the spleens of WT B6.$Cre^-VDR^{fl/fl}$ mice and B6.$Cre^+VDR^{fl/fl}$ mice with $CD4^+$ T-cell-specific *Vdr* targeting, whether or not they were treated with calcitriol (184). These data suggest fully differentiated, mature rodent $CD4^+Foxp3^+$ T cells may not be calcitriol responsive. Contrary to this view, VDR-dependent down-regulation (187) and up-regulation (185) of *Foxp3* transcription in rodent $CD4^+Foxp3^+$ T cells have both been reported. It will be interesting to learn the molecular details of *Vdr* and *Foxp3* gene expression control as research in this area progresses.

CD4+ T CELLS ARE CALCITRIOL TARGETS IN AUTOIMMUNE DISEASE

Selective *Vdr* gene inactivation experiments in rodents provided unequivocal evidence that calcitriol targets $CD4^+$ T lymphocytes for the purpose of immune system regulation (184). Reciprocal mixed bone marrow chimera studies established that hematopoietic cell *Vdr* gene expression was necessary for calcitriol to inhibit EAE induction. In fact, chimeric mice lacking the VDR in hematopoietic cells had a particularly aggressive EAE disease course. Subsequently, mice with $CD4^+$ T-cell-specific *Vdr* targeting were constructed. Evaluation of T-cell subsets in the periphery of naïve T-cell *Vdr*-targeted mice assured that this genetic manipulation did not influence T-cell proportions during thymic selection. Neither vitamin D_3 nor calcitriol inhibited EAE induction in mice with $CD4^+$ T-cell-specific *Vdr* targeting (184). Thus, EAE data establish that calcitriol exerts protective biological effects against autoimmunity *in vivo* through the nuclear VDR in $CD4^+$ T lymphocytes. These data do not rule out vitamin D effects on myeloid cells for a pathogen protective immune response (188). Studies in mice with $CD4^+$ T-cell-specific *Vdr* targeting (184), and in humans with loss-of-function mutations in the *VDR* gene (189) support the view that *the outcome of calcitriol signaling within CD4+ T cells is likely a major determinant of sunlight's influence on the emergence of an autoimmune disease phenotype.*

VITAMIN D AND THYMOCYTE SELECTION

THYMIC NEGATIVE SELECTION IN AUTOIMMUNE DISEASE

The TCR repertoire is shaped in the mammalian postnatal thymus, and autoimmune disease risk is believed to reflect in part defects in this process (190). When immature $CD4^+CD8^+$ thymocytes engage cortical thymic epithelial cells expressing self peptides embedded in major histocompatibility complex (MHC) molecules, those that bind self MHC molecules with adequate affinity undergo positive selection and develop into $CD4^+$ or $CD8^+$ T cells. Those that bind with inadequate affinity for survival signaling undergo apoptosis. Surviving thymocytes migrate to the thymic medulla where they engage medullary thymic epithelial cells (mTEC) presenting self peptides, including peptides derived from tissue-restricted proteins. Cells that bind self peptides with high affinity receive apoptotic signals. The survivors are released to form the pre-immune TCR repertoire.

An analysis of human autoimmune TCR–peptide–MHC complexes revealed structural anomalies compared to anti-microbial TCR–peptide–MHC complexes suggesting these TCR may have escaped thymic negative selection (191). For example, a TCR from an MS patient recognized both a DRB1*1501-restricted MBP peptide and a DRB5*0101-restricted EBV peptide, suggesting this TCR may have escaped negative selection become activated by a pathogen (192). A negative selection failure during early life is believed to release potentially pathogenic T cells that initiate autoimmune disease when a cross-reactive pathogen peptide activates them (116).

VITAMIN D AND THYMIC NEGATIVE SELECTION

Sunlight and vitamin D_3 exert their strongest influence on MS risk during childhood, which coincides with the peak period for T-cell selection in the postnatal thymus (19). Intriguingly, recent data quantifying signal joint TCR excision circles (sjTRECs) in human cord blood T cells as a function of season demonstrated seasonal variability in thymic output (193). May-born infants had significantly lower circulating 25-OHD_3 and higher sjTRECs/10^5 T cells than November-born infants. The sjTRECs are extra-chromosomal DNA circles formed during TCR gene rearrangement. They are not replicated as mature peripheral T cells divide, so they serve as markers of recent thymic emigrants in the peripheral T-cell repertoire. The data suggest a positive influence of 25-OHD_3 on thymic negative selection. Rigorous investigation of this possibility is warranted because of the significance such an influence would have on autoimmune disease risk acquisition, and the insight it would provide into the timing of risk acquisition and therefore the timing of etiology-based intervention strategies to prevent disease.

VITAMIN D AND CD4+ Th1 AND Th17 T CELLS

EFFECTOR T-CELL ANTI-MICROBIAL RESPONSE, CROSS-REACTIVITY, AND AUTOIMMUNE DISEASE

Exposure to common infectious or commensal organisms triggers activation of those $CD4^+$ T cells capable of recognizing foreign antigens in an MHCII context. The biological imperative to provide adequate immune cover for the host inevitably results in some effector T-cell cross-reactivity between foreign and self peptides due to molecular mimicry (116). Peptide antigen diversity is orders of magnitude larger than TCR diversity (194), and TCR–peptide–MHC binding shows conformational plasticity, relatively low affinity, and rapid off-kinetics (191, 195). The immune system has evolved mechanisms to prevent overly aggressive T-cell-mediated responses that cross-react with host peptides from damaging host tissue (196). These mechanisms include inhibition of pro-inflammatory cytokine synthesis, and Treg cell-mediated restriction of T-cell expansion through inhibitory receptors like cytotoxic T-lymphocyte antigen 4 (CTLA4), induction of T-cell anergy, and cell death signaling (197). Intriguing new research has documented a central role for the vitamin D system in the development of Treg cells and the termination of effector T-cell responses.

MODELING HUMAN AUTOIMMUNE DISEASE

Two animal models have been particularly useful in developing mechanistic knowledge of direct calcitriol actions in $CD4^+$ T cells, murine EAE (198, 199), and the NOD mouse (200). These animal models show strong parallels to their respective human diseases, especially in the immunological aspects of disease. The parallels are imperfect, for example regarding the role of human microbial exposures, because our understanding of human autoimmunity is incomplete. Nevertheless, hypothesis testing is more facile in animal models where direct manipulation of contributing factors is possible. Calcitriol inhibited autoimmune disease in the EAE and NOD models (201–203). Working back and forth between animal models and human disease has allowed rapid forward progress to be made in understanding how the vitamin D system modulates immunity.

VITAMIN D AND Th1 CELLS

Disease prevention studies in animal models have suggested that calcitriol directly inhibits encephalitogenic Th1 cells. Calcitriol inhibited EAE induction (182, 183, 185, 202–205), and targeting the *Vdr* gene specifically in $CD4^+$ T cells abrogated this inhibition (184). However, calcitriol had no effect on $CD4^+$ T-cell priming in the periphery, myelin-specific T-cell trafficking into the CNS, or IFN-γ synthesis by freshly explanted, purified $CD4^+$ T cells from EAE mice (182). Instead, the CNS-infiltrating myelin-specific $CD4^+$ T cells displayed an anergic phenotype in the calcitriol-treated mice. These findings have been confirmed (171, 184). In another strain, calcitriol inhibited EAE induction, but the mechanism reported was a decreased Th1 cell frequency in peripheral lymphoid tissues (206).

The mechanisms involved in amelioration of established autoimmune disease may differ from those involved in autoimmune disease prevention. Administering calcitriol to rodents with established EAE resulted in very rapid disease remission that correlated with fewer CNS $CD4^+$ T cells, loss of IFN-γ-production, and a significant decrease in CNS pathology (207–210). Induction of Treg cells in the EAE studies is discussed below.

A short course of oral calcitriol treatment in healthy male volunteers had no measurable effect on circulating IFN-γ (211). Likewise, in two studies carried out in MS patients, vitamin D_3 supplementation had no measurable effect on circulating IFN-γ (96, 212). In a third study performed in healthy volunteers, vitamin D_3 supplementation decreased the mean percentage of Th1 cells in the circulation from 20 to 17% (213).

VITAMIN D AND Th17 CELLS

Disease prevention studies in animal models have also suggested that calcitriol directly inhibits encephalitogenic Th17 cells. Experimental autoimmune uveitis (EAU) induced by immunization of mice with a retinal antigen serves as model for human autoimmune uveitis. Oral calcitriol prevented as well as partly reversed EAU disease (214). In this model, calcitriol treatment *in vivo* of mice impaired T-cell commitment to the Th17 lineage as well as Th17 production of IL-17.

Similar results were reported in the EAE model. The spleens of calcitriol-treated mice had fewer splenic Th17 cells and lower IL-17 production than the placebo controls (183, 187). Mice with global inactivation of the *Vdr* gene had activated Th17 cells that overproduced the cytokine (215). Calcitriol treatment reduced Th17 cells in the CNS in an EAE prevention study (171) and in two EAE treatment studies (185, 216). Different mechanisms were suggested to explain these findings (**Figure 1A**). In one study, calcitriol did not suppress *Il17* gene transcription, but inhibited Th17 cell IL-17F production in a VDR-dependent manner by a post-transcriptional mechanism (183). In another study, calcitriol suppressed *Il17* gene transcription by blocking nuclear factor for activated T cells (NFAT), recruiting histone deacetylase, and sequestering Runt-related transcription factor 1 (Runx1) (185). In the third study, elimination of Th17 cells by a programed cell death mechanism was suggested (216). The reconciliation of these divergent mechanisms awaits further investigation.

Studies of calcitriol and Th17 cell activity in humans are at an early stage. Cell culture studies have shown that adding calcitriol to

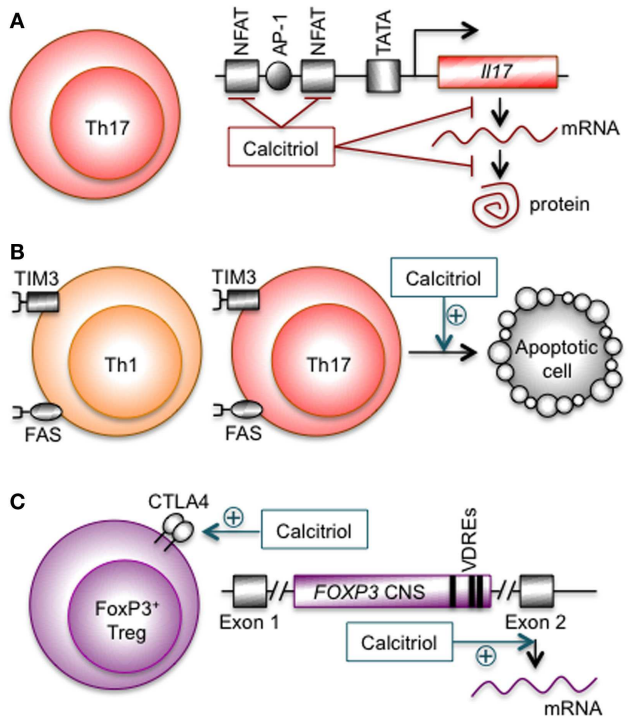

FIGURE 1 | Proposed calcitriol mechanisms in effector Th1, Th17, and FoxP3+ Treg cells. **(A)** Calcitriol and IL-17 synthesis in Th17 cells. Calcitriol inhibited IL-17 synthesis in a VDR-dependent manner by a post-transcriptional mechanism (183, 187). It also blocked NFAT, recruited histone deacetylase, and sequestered Runx1 to suppress murine *Il17* gene transcription (185). **(B)** Calcitriol and effector CD4+ T-cell apoptosis. Calcitriol increased CD4+ T-cell sensitivity to extrinsic cell death signals that may have been transduced through the FAS ligand–FAS–caspase-8 and/or the galectin-9–TIM3–calpain pathways (208–210, 216). **(C)** Calcitriol and *FOXP3* gene transcription. Calcitriol increased the transcription of the murine *FoxP3* (185) and human *FOXP3* genes (217). Three VDREs that functioned as calcitriol-dependent enhancers were identified in a conserved non-coding sequence (abbreviated CNS in this diagram) between exons 1 and 2. Calcitriol also increased CTLA4 protein expression (218–220).

activated CD4+ T cells reduced the frequency of Th17 cells (218, 221), an effect that was more pronounced in cultures of T cells from women than from men (172). New data have extended these observations in a vitamin D_3 dose escalation study performed in healthy controls during the UV-restricted winter months (213). As supplementary vitamin D_3 increased from 2000 to 8000 IU/day, the mean serum 25-OHD rose from 30 ± 12 to 159 ± 29 nmol/L, and the proportions of Th1 and Th17 cells in the peripheral blood decreased. There appeared to be a threshold effect with at least 70 nmol/L of 25-OHD triggering a mean 40% decrease in circulating Th17 cells in the healthy controls. Other studies found no correlation between Th17 cells and 25-OHD in MS patients (222), and no effect of supplementary vitamin D_3 on Th17 cells in MS patients (223). Why the human data are conflicting is not clear, but vitamin D_3 status at enrollment, vitamin D_3 dose, dose frequency (135), use of disease-modifying drugs, and timing of sampling relative to estrogen cycling in women are potential confounding factors. Nevertheless, given the strong animal modeling data and some human *in vivo* data that are consistent with animal modeling, it is reasonable to suggest that *vitamin D may significantly influence the emergence of an autoimmune disease phenotype by dampening pathogenic Th17 cells and IL-17 synthesis.*

VITAMIN D AND Th2 CELLS

Analyses of Th2 cells *in vivo* have also yielded inconsistent results. Administering calcitriol to mice before EAE induction increased the IL-4 transcripts in the lymph nodes and in the CNS compared to the placebo controls (204). Also, targeted disruption of the *IL-4* gene moderately decreased the protective function of calcitriol in EAE (224). A subsequent report found no significant differences between calcitriol-pretreated and placebo-pretreated B10.PL mice with regard to IL-4 mRNA in the lymph nodes or the CNS after immunization with MBP (182). There was also no effect of calcitriol on the IL-4 protein synthesis per Th2 cell. Another report found administering calcitriol to Biozzi AB/H mice before EAE induction had no effect on the IL-4-producing Th2 cell frequency (206). Thus, there are some inconsistencies regarding IL-4 that remain to be resolved. Since calcitriol inhibition of EAE decreased slightly in *IL-4*-null mice, there may be some IL-4 contribution to the mechanism (224), but it is possible that Th2 cells were not the source of the protective IL-4.

The IL-5-producing Th2 cells have a pathogenic role in human asthma. When CD4+ T cells from asthma patients were stimulated with dust mite allergen in the presence of calcitriol, the hormone decreased IL-5, IL-9, and IL-13 production (225). Calcitriol's actions to promote IL-10-producing Treg cells in asthma patients are discussed below.

VITAMIN D AND CD4+ T-CELL PROGRAMED CELL DEATH

EFFECTOR T-CELL APOPTOSIS AND AUTOIMMUNE-MEDIATED TISSUE DAMAGE

When effector CD4+ T cells are no longer needed for antimicrobial defense, programed cell death mechanisms remove them to limit immune-mediated tissue damage. Several mechanisms used by CD4+ Treg cells (197, 226), astrocytes and neurons (227, 228), and a few other types of cells to control effector CD4+ T cells involve apoptosis induction. In individuals predisposed to autoimmunity, it appears that effector CD4+ T cells resist cell death mechanisms, proliferate, and continue to produce inflammatory molecules that damage the host's tissues.

The effector T lymphocytes from the T1D-susceptible NOD mice were resistant to cell death signals. The resistance to cell death mapped to a genomic region encompassing the *Ctla4* gene, which was defectively expressed on T cells from these mice (229). In human autoimmunity, myelin-specific CD4+ T cells from MS patients provide an example of defective cell death mechanisms (230). These T cells were defective in the FAS–caspase 8 cell death pathway (231) and the galectin-9–TIM-3 cell death pathway (232). It was intriguing that myelin-specific T cells from MS patients with benign disease had a significantly augmented galectin-9–TIM-3 cell death pathway compared to MS patients with active disease (Saresella et al., ECTRIMS 2011, Abstract P324). Moreover, MS disease activity fluctuated seasonally, with a high frequency of new lesions following a period of reduced ambient UV light and *vice versa* (31). Taken together, the seasonal fluctuations in

MS disease activity and disease activity-associated fluctuations in T-cell apoptosis resistance hint at a possible causal relationship between ambient UVB light, vitamin D_3 supplies, myelin-specific T-cell susceptibility to cell death, and demyelinating disease activity.

VITAMIN D REGULATION OF EFFECTOR T-CELL APOPTOSIS

A causal relationship between calcitriol, myelin-specific T-cell responsiveness to cell death signals, and demyelinating disease activity has been demonstrated in the animal model of MS (**Figure 1B**). In animals with EAE, calcitriol treatment *in vivo* increased the susceptibility of pathogenic myelin-specific $CD4^+$ T cells to extrinsic, CNS-derived apoptotic signals (207). This treatment induced the pro-apoptotic gene encoding caspase-8-associated protein, which is essential for FAS-mediated apoptosis, and repressed cellular inhibitor of apoptosis protein 2 (cIAP-2), an apoptosis inhibitor (209). Effector T-cell death is triggered in the CNS by astrocyte- and neuron-mediated activation of the T-cell FAS death pathway (227, 228, 233–235). In the calcitriol treatment study, apoptotic $CD4^+$ T cells were evident in CNS lesions and IFN-γ production ceased by 12 h post treatment; within 1 day, the $CD4^+$ T-cell numbers were 60% reduced, correlating with abatement of clinical disease signs by day 3 (208, 210). These T-cell changes were not observed in the periphery or *in vitro*. A transient increase in $Helios^+FoxP3^+$ Treg cells coincided with the loss of effector T cells suggesting the Treg cells may have played an active role in effector T-cell elimination (216). In the NOD model of T1D, peripheral T cells were resistant to programed cell death signals, and calcitriol treatment restored their sensitivity to these signals (229, 236–238). These results support a direct calcitriol action on effector T cells to promote sensitivity to apoptotic signals.

Vitamin D may also influence other cell death pathways in effector $CD4^+$ T cells. Treg cells expressing galectins can trigger effector T-cell death via the TIM3 pathway (226). The N-linked glycans, in particular terminal galactosyl residues, are essential for galectin-9 binding to TIM3 and induction of cell death through calpain and caspase-1 activation (239, 240). Importantly, calcitriol enhanced α-mannosidase *N*-acetyl-glucosaminyl transferase 1, the rate-limiting enzyme in N-linked glycan synthesis (241), UDP-galactose: beta *N*-acetyl glucosamine-beta-1,3-galactosyl transferase, and calpain (209). Thus, calcitriol may improve T-cell sensitivity to the galectin-9–TIM3–calpain cell death pathway, which was impaired in MS patients (232). Together, the animal modeling and human *in vivo* data linking vitamin D and calcitriol with effector T-cell apoptosis suggest that *vitamin D may significantly influence the emergence of an autoimmune disease phenotype by increasing effector $CD4^+$ T-cell sensitivity to extrinsic cell death signals.*

VITAMIN D AND $CD4^+$ T-REGULATORY LYMPHOCYTES

TREG LYMPHOCYTES IN AUTOIMMUNE DISEASE

$CD4^+$ Treg cells are defined functionally by their ability to limit prolonged effector T-cell activation, thereby preventing autoimmune-mediated pathology (242). T lymphocytes that suppress autoimmunity were first demonstrated in mice (243–246). Subsequently, their existence was confirmed in humans (247). Several types of Treg cells exist differing in origin, phenotype, and function. The most intensely studied are those for which FoxP3 serves as the definitive transcription factor in lineage specification (248). One $FoxP3^+CD4^+$ Treg cell subset arises during thymic development (tTreg), whereas another arises during TCR engagement in the periphery (pTreg) (249). These $FoxP3^+CD4^+$ Treg cell subsets are non-functional in male *scurfy* mice and boys with the multi-organ autoimmune disease immune-dysregulation polyendocrinopathy enteropathy X-linked syndrome (IPEX), due to loss-of-function mutations in the X-linked rodent *Foxp3* and human *FOXP3* genes, respectively. The FoxP3 protein serves as a biomarker for this Treg lineage.

The T-regulatory cell type 1 (Tr1) cells differ from $FoxP3^+CD4^+$ Treg cells in origin, phenotype, and function (250). The $CD4^+$ Tr1 cells are peripheral memory T lymphocytes that are anergic and do not express FoxP3 (251). Selective biomarkers for Tr1 cells in humans and rodents are co-expression of integrin alpha 2 subunit (CD49b, a cell adhesion molecule) and lymphocyte activation gene 3 (LAG-3 or CD223, a negative regulator of effector T-cell function) (252). Upon CD3 stimulation and co-stimulation via the complement regulator CD46 in the presence of IL-2, the $CD4^+$ Tr1 cells rapidly proliferate and secrete large amounts of IL-10 and TGF-β to exert suppressive function (253). The Tr1 cells also release granzyme B to specifically lyse APC of myeloid origin, effectively terminating further effector T-cell activation.

Functional defects in $FoxP3^+CD4^+$ Treg cells have been described in multiple autoimmune diseases (254). Such defects were first reported and confirmed in peripheral blood T cells from MS patients (255, 256). Additional evidence emerged for reduced $CD4^+$ Treg cell generation in the thymus of MS patients (257, 258). The impairment correlated with reduced FoxP3 expression in MS patient T cells (259, 260). Studies of $FoxP3^+CD4^+$ Treg cell defects in MS have been reviewed (261, 262). Functional defects in $FoxP3^+CD4^+$ Treg cells have also been extensively described in T1D (263). Below, we review research suggesting calcitriol enhances the expression of Helios, a positive regulator of FoxP3, and of FoxP3 itself.

Functional defects in IL-10-producing $CD4^+$ Tr1 cells have also been described in many autoimmune diseases (250). An early report demonstrated that T cells from T1D patients showed extreme polarization toward a pro-inflammatory Th1 phenotype, whereas the T cells from the non-diabetic, HLA-matched control subjects showed an extreme bias toward the IL-10-secreting Treg phenotype (264). Functional defects in Tr1 cells have also been extensively studied in MS. Stimulation of MS patient T cells *in vitro* via CD3 and CD46 elicited very little IL-10 production compared to controls (265). These findings have been confirmed (261, 266, 267) and extended to a primate model of MS (268). Functional defects in IL-10-producing $CD4^+$ Tr1 cells have also been described in asthma and rheumatoid arthritis (269). Below, we review research suggesting calcitriol enhances the expression of IL-10 and controls the Th1–Tr1 switch through modulation of CD46, a positive regulator of IL-10.

CUTANEOUS UVB LIGHT EXPOSURE AND Treg LYMPHOCYTES

Cutaneous UVB light exposure and *in situ* vitamin D metabolism may have a particularly important role in cutaneous Treg cell development. Cutaneous UVB exposure promotes T-cell-mediated

immunity to microbial pathogens; Finsen received the 1903 Nobel Prize in Medicine for phototherapy of skin diseases (270). More recently, the role of cutaneous UVB exposure in peripheral immune tolerance has been recognized (271). The dual role of UVB exposure illustrates how the vitamin D system may have evolved to promote anti-pathogen responses at environmental interfaces, and subsequently to terminate these responses before the tissues sustain immune-mediated damage.

Ground-breaking animal modeling studies revealed how cutaneous UVB exposure promotes peripheral immune tolerance at the molecular level (272). Infection increased keratinocyte expression of receptor-activator of NF-kappaB ligand (RANKL). In transgenic mice with keratinocytes overexpressing RANKL, the keratinocytes signaled receptor-activator of NF-kappaB (RANK)-expressing Langerhans cells to induce IL-10-producing pTreg cells in the skin-draining lymph nodes. These pTreg cells suppressed the contact hypersensitivity response to cutaneous antigens in the original host animal and in adoptive transfer host animals. Furthermore, when skin was grafted from WT mice or *Tnfrsf11a*-null mice lacking RANKL onto WT host animals, UVB irradiation of the WT but not the *Tnfrsf11a*-null skin graft prevented graft rejection. These experiments established that keratinocyte RANKL expression was necessary for UVB exposure to induce IL-10-producing pTreg cells specific for the grafted skin to maintain peripheral tolerance.

Importantly, cutaneous UVB exposure promoted pTreg cell development by a calcitriol and VDR-dependent mechanism (273). Keratinocytes have a complete vitamin D_3 biosynthetic pathway which when stimulated by infection, injury, or light produced 2–5 nmol/L of calcitriol *in situ* (159, 274), about 20- to 50-fold higher than calcitriol in the blood plasma of non-pregnant women (275). Furthermore, calcitriol transcriptionally activated the murine (*Tnfrsf11a*) and human (*TNFRSF11A)* genes encoding RANKL through highly evolutionarily conserved and functionally active VDREs in the promoter regions, as described in osteoblasts (276–278), murine and human T lymphocytes (279, 280), and keratinocytes (127). In the animal model of cutaneous hypersensitivity, topically applied calcitriol mimicked cutaneous UVB exposure by inducing keratinocyte RANKL expression and stimulating IL-10-producing pTreg cells by a mechanism that was VDR-dependent (281, 282). Cell labeling studies demonstrated that the pTregs trafficked between the skin, the skin-draining lymph nodes, and the circulation (283). Calcitriol suppressed T-cell expression of the gut-homing receptors and increased T-cell expression of CC chemokine receptor 10, enabling T-cell migration to the skin-specific chemokine CCL27 produced by keratinocytes (100). In summary, seminal studies of experimental UVB-induced peripheral tolerance in rodents established that a calcitriol- and VDR-dependent pathway exists involving (i) keratinocyte synthesis of calcitriol and stimulation of RANKL expression, (ii) signaling to RANK-expressing Langerhans cells, (iii) movement of the Langerhans cells to the skin-draining lymph nodes, (iv) induction of cutaneous antigen-specific, IL-10-producing pTreg cells, and finally (v) movement of the pTregs to the skin to prevent cutaneous antigen-specific effector T cells from degrading the epidermal barrier.

New research has begun to translate this knowledge to humans. Subjects from the North of Scotland who had skin disease were recruited between December and March for a phototherapy study (284). The subjects had a mean baseline 25-OHD level of 34 nmol/L and 0.5% $FoxP3^+$ pTreg cells as a percentage of blood $CD3^+$ T cells. After 4 weeks of phototherapy, the mean 25-OHD was 78 nmol/L and the $FoxP3^+$ pTreg cell percentage was 1.6%. Another study demonstrated that UVB exposure substantially expanded pTreg cell numbers in the skin, and these skin pTreg cells appeared to have derived from peripheral immune organs (285). The studies in animals and humans implicating calcitriol as a positive regulator of the *Tnfsf11a* and *Tnfrsf11a* genes and cutaneous $FoxP3^+$ pTreg cell induction suggest that *during periods of light starvation, phototherapy may be a particularly efficient method of influencing the emergence of an autoimmune disease phenotype by increasing $CD4^+CD25^+FoxP3^+$ Treg cell development and suppressive function.*

VITAMIN D AND THE IL-10–IL-10R PATHWAY

Interleukin-10 is a particularly important anti-inflammatory cytokine that protects the CNS (286), the airway (287), the gastrointestinal tract (288), and other tissues from immune-mediated pathology (289, 290). Disruption of the IL-10–IL-10R signaling pathway results in severe inflammatory disease (291).

Early research in two rodent models suggested that calcitriol promotes the development of IL-10-producing Treg cells. In the EAE model, suppressor T cells inhibited disease by an IL-10-dependent mechanism (292, 293). Calcitriol inhibited EAE not by blocking T-cell priming in the periphery or effector T-cell trafficking into the CNS, but instead by inducing *Rag-1* gene-dependent regulatory lymphocytes (182). Subsequent work demonstrated that activation of human and mouse T cells in the presence of dexamethasone and calcitriol *in vitro* yielded IL-10-producing Tr1 cells (294). Furthermore, vitamin D_3 or calcitriol-mediated inhibition of EAE required functional *Il10* and *Il10R* genes, bidirectional IL-10–IL-10R signaling between hematopoietic and non-hematopoietic cells (205), and a functional *Vdr* gene in $CD4^+$ T cells (184). Calcitriol had no effect on IL-10R expression (205). In the NOD model of T1D, calcitriol increased the frequency of Treg cells in the pancreatic lymph node (295), and global *Vdr* gene inactivation decreased the frequency of these cells (296). A positive calcitriol influence on T-cell IL-10 synthesis was observed *in vitro* (297). These animal studies suggested calcitriol may inhibit autoimmune disease at least in part through a VDR-dependent action on $CD4^+$ T cells to induce IL-10-producing Treg cells.

There is limited information on a possible direct link between vitamin D and IL-10 in humans. An early study found a seasonal variation in the cord blood IL-10 level that correlated directly with the 25-OHD for infants born at 51°N (298). The 25-OHD levels were 99% higher and IL-10 was 43% higher in samples obtained in the summer compared to samples obtained in the winter. Investigations of calcitriol effects on IL-10 synthesis by human T cells activated *in vitro* have yielded conflicting results with reports of both enhancement (172, 221) and inhibition (179). A very interesting recent study examined the effect of adding 25-OHD_3 to cultures of lymphocytes from healthy controls and from hereditary vitamin D-dependent rickets (HVDRR) patients with loss-of-function mutations in the *VDR* gene (189). The 25-OHD_3 increased IL-4, IL-10, and IFN-γ production from

control lymphocytes, but not patient lymphocytes, demonstrating calcitriol synthesis and VDR dependence of IL-10 induction *in vitro*.

VITAMIN D AND Treg CELLS IN ASTHMA

Asthma research has yielded important new insights regarding vitamin D and Treg cells (299). One of them relates to the balance between immunity to pathogens and self-tolerance. Calcitriol ingestion by healthy volunteers and calcitriol addition to cultures increased $CD4^+$ T-cell IL-10 secretion and TLR9 expression (300). Adding TLR9 agonists to the cultures decreased T-cell IL-10 production and suppressive function. These findings are significant because they suggest pathogen signaling through TLR9 could suspend T-cell suppressive function temporarily until an infection has been cleared. A second insight relates to calcitriol enhancement of T-cell surface molecules that dampen immune responses. In this case, calcitriol up-regulated CD200 on human peripheral and respiratory tract $CD4^+$ T cells *in vitro*, and there was a trend toward up-regulation *in vivo* in healthy, but not asthmatic individuals (301). CD200 is an immunoglobulin superfamily member; it imparts a unidirectional negative signal to suppress innate and adaptive immune responses.

Yet another insight from the asthma research relates to the distinction between $CD4^+FoxP3^-IL\text{-}10^+$ Tr1 cells and $CD4^+FoxP3^+IL\text{-}10^-$ Treg cells. Systemic vitamin D status correlated directly with airway levels of IL-10 and $CD4^+FoxP3^+$ T cells in pediatric asthma patients and in healthy controls (302, 303). In cultures of human peripheral blood T cells, adding calcitriol at moderate levels (10^{-8} mol/L) increased the frequency of $CD4^+IL\text{-}10^+$ Tr1 cells, whereas higher calcitriol (10^{-6} mol/L) increased the frequency of $CD4^+FoxP3^+$ T cells. However, there was little co-expression of FoxP3 and IL-10, and IL-10 impaired calcitriol-mediated enhancement of FoxP3. Modulating the cytokine environment to include TGF-β together with calcitriol favored $CD4^+FoxP3^+$ T-cell outgrowth (304). The $CD4^+IL\text{-}10^+$ and $CD4^+FoxP3^+$ T-cell populations had equivalent suppressive activity *in vitro*, although their suppressive mechanisms were IL-10-dependent and IL-10-independent, respectively.

These data are highly significant for two reasons. First, they demonstrate that the vitamin D system supports two phenotypically and functionally distinct Treg cell populations. Secondly, they suggest that as infections are resolved, calcitriol-supported development of $CD4^+FoxP3^-IL\text{-}10^+$ Tr1 cells and $CD4^+FoxP3^+IL\text{-}10^-$ Treg cells may proceed in an ordered sequence to perform slightly different functions, for example attenuating innate and adaptive immune responses by IL-10-dependent and IL-10-independent mechanisms, respectively. Many interesting questions remain regarding the underlying mechanisms of Treg cell immune response regulation in the airway, and the generality of these findings to other interfaces between the environment and the host organism where immunity to pathogens must be balanced with self-tolerance.

VITAMIN D AND FOXP3+ Treg LYMPHOCYTES

Above, we summarized early animal modeling data suggesting calcitriol promoted the development of IL-10-producing Treg cells before FoxP3 expression was in use as a Treg biomarker. New animal modeling data suggest calcitriol may be a positive regulator of the murine *FoxP3* gene itself (185), and of the *Ikzf2* gene encoding Helios (216), a transcription factor that binds to the *FoxP3* promoter and stimulates its transcription (305–307).

$CD4^+Foxp3^+$ Treg cell studies in humans are scarce and conflicting. Two reports correlated 25-OHD with circulating $CD4^+Foxp3^+$ Treg cell percentages (308, 309). One study correlated the suppressive capacity of the $CD25^+CD4^+$ Treg cells with serum 25-OHD levels in MS patients (222), but this association could not be substantiated upon vitamin D_3 supplementation (223). A more recent study correlated seasonal increases in 25-OHD and calcitriol in healthy men with increased Treg cell Foxp3 expression, a drop in memory $CD4^+CD45RO^+$ T cells, a reduced capacity for T-cell pro-inflammatory cytokine production, and increased CCR4, CCR6, CLA, CCR9, and CCR7 levels for homing to skin, gut, and lymphoid tissue (310). Seemingly contradictory results were reported for infants whose 25-OHD levels and T-cell subsets in cord blood showed no correlations (311). Potential confounders in human studies are differences in phenotypic markers used in the analyses, vitamin D_3 status when beginning vitamin D_3 supplementation, supplementation protocols (135), use of disease-modifying drugs in patients, and timing of sampling relative to estrogen cycling in women.

Examining human T cells in culture documented an influence of calcitriol on FoxP3 (**Figure 1C**). Activation of human $CD4^+CD25^-$ T cells *in vitro* in the presence of calcitriol resulted in greater numbers of $CD4^+CD25^+FoxP3^+$ Treg cells (221), particularly for female T cells (172). Addition of calcitriol to human $CD4^+CD25^-$ T-cell cultures increased CTLA4 (CD152) and FoxP3 protein expression, the latter requiring the presence of IL-2 (218). The increase in CTLA4 and FoxP3 protein expression was confirmed for MS patient T cells (219), and the CTLA4 increase was confirmed for T1D patient T cells (220). The suppressive function of the calcitriol-induced $CD4^+CD25^+FoxP3^+$ Treg cells was confirmed in many reports (218, 220, 221, 312).

Calcitriol positive regulation of CTLA4 is critically important, because this protein is an essential negative regulator of immune responses (313, 314). Humans who were heterozygous for loss-of-function mutations in the *Ctla4* gene developed a highly penetrant autoimmune syndrome with strong similarity to IPEX. Specifically, they displayed loss of $CD4^+FoxP3^+$ Treg cell suppressive function, hyperactivation of effector T cells, lymphocyte infiltration of multiple target organs, and fatal autoimmune disease. CTLA4 strips CD80 and CD86 from the APC by endocytosing and degrading these proteins to block their co-stimulation of CD28 on T cells (315). Calcitriol enhancement of this mechanism may account in part for the hormone's actions as an immune response terminator.

Intriguing new data complemented the rodent data (185) and revealed that calcitriol increased human *FOXP3* gene expression by a transcriptional mechanism (217). The human *FOXP3* and murine *Foxp3* genes share homology in a conserved non-coding sequence (+1714 to +2554 relative to the *FOXP3* transcriptional start site) that functions as an enhancer (316). Within this enhancer region, investigators identified three VDREs that enhanced promoter activity in a calcitriol-dependent manner (217). Moreover, T-cell stimulation through CD3 and CD28 in

the presence of IL-2 and calcitriol generated $CD4^+CD25^+FoxP3^+$ Treg cells that inhibited target $CD4^+$ T-cell proliferation by a cell contact- and FOXP3-dependent mechanism. Collectively, the data demonstrating calcitriol enhancement of *Ikzf2*, *FOXP3*, and *Ctla4* gene expression suggest that *vitamin D may significantly influence the emergence of an autoimmune disease phenotype by increasing $CD4^+CD25^+FoxP3^+$ Treg cells and/or their suppressive function.*

VITAMIN D, CD46, AND IL-10-PRODUCING Tr1 LYMPHOCYTES

Important new research into the function of CD46 has demonstrated the existence of a calcitriol-mediated Th1–Tr1 switch controlling the delicate homeostatic balance between the pro-inflammatory and anti-inflammatory states (**Figure 2**). CD46 and its murine analog complement receptor 1-related protein Y (Crry) are transmembrane glycoproteins that bind complement fragments C3b and C4b, and function as T-cell costimulatory molecules (269). Temporal processing of two alternatively spliced CD46 cytoplasmic tails, Cyt1 and Cyt2, served as a molecular rheostat controlling activation and de-activation of human T-cell responses (317). Co-engagement of the TCR and CD46 first enhanced Th1 cell effector function (318). However, as IL-2 accumulated, CD46 cytoplasmic domain interactions with the serine–threonine kinase SPAK promoted effector Th1 cell switching to a Tr1 phenotype with low IL-2 production, high IL-10 production, and the capability to suppress bystander T-cell activation. Thus, CD46 served as a molecular link between complement activation and a Th1–Tr1 switch for immune response termination (269). This switch was defective in MS (265, 266), asthma (319), and rheumatoid arthritis patient T cells (318), effectively locking the immune response in an interminable pro-inflammatory state.

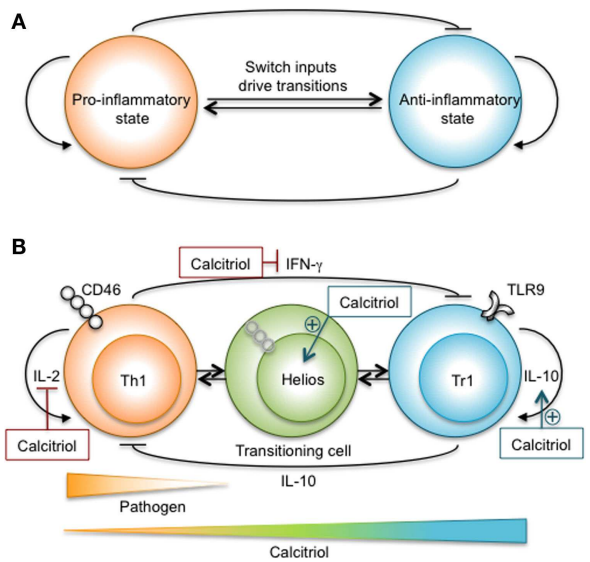

FIGURE 2 | A proposed Th1–Tr1 bi-stable biological switch bridging two immune states, pro-inflammatory and anti-inflammatory.
(A) A theoretical diagram of two opposing immune states [adapted from a generalized bi-stable switch diagram (302)]. Each circle represents T cells that are in a stable biological state characterized by a particular gene regulatory network and capable of responding to external stimuli in a biologically appropriate manner. Biological barriers impede transitions between the pro- and anti-inflammatorystates. Each biological state is capable of reinforcing its own state, denoted as lines terminating in arrows, and inhibiting the opposing state, denoted as blocked lines. External stimuli, for example pathogens, hormones, cell-cell interactions, and soluble mediators serve as switch inputs to drive a transition. For example, signals 1 and 2 can be organized as "1 or 2", "1 and 2", or "1 not 2" to trigger a dynamic transition between the two states. **(B)** The proposed Th1–Tr1 switch appears to have the qualities of a bi-stable biological switch. The Th1 cytokine IFN-γ and the Tr1 cytokine IL-10 impede the development of Tr1 and Th1 cells, respectively. The autocrine growth factors IL-2 and IL-10 reinforce the survival and proliferation of Th1 and Tr1 cells, respectively. Diminishing pathogen, sensed through TLR molecule engagement (280), maturation of the pathogen-specific immune response, sensed through CD46 molecule engagement by C3b and C4b (299, 300), and accumulating calcitriol in the microenvironment, sensed through the VDR in Th1 cells, provide the external stimuli to alter the dynamics of the system and drive the transition to the Tr1 biological state. Helios may be a biomarker of effector T cells undergoing a transition to a tolerant state (183). Calcitriol increased Helios in $CD4^+$ T cells at the beginning of a calcitriol-induced EAE disease remission (144), and was recently identified as an inhibitor of *Il2* gene transcription by an epigenetic silencing mechanism (184).

Calcitriol profoundly amplified the Th1–Tr1 switch *in vitro* in T cells from healthy donors and patients with MS (219). Calcitriol also amplified this switch *in vitro* in T cells from T1D patients, imprinting the T cells with an IL-10-producing Tr1 phenotype, and modulating surface expression of chemokine receptors to enable homing to inflamed sites (220, 312). Determining whether this switch functions *in vivo* is a high priority goal. The emerging data suggest that *vitamin D may significantly influence the emergence of an autoimmune disease phenotype by controlling a Th1–Tr1 switch that provides a bridge between two apparently stable immune states, pro-inflammatory and anti-inflammatory.* The calcitriol-driven Th1–Tr1 switch is of utmost importance to autoimmune disease, because each state appears to be self-reinforcing and capable of blocking the opposing state (**Figure 2**). Multistable switches are known to play an important role in hematopoietic cell fate decisions controlled by gene regulatory networks (320), and hormones are known to be of paramount importance in controlling these networks.

Calcitriol enhancement of the transcription factor Helios may also be important to the Th1–Tr1 switch. Calcitriol treatment transiently induced $CD4^+Helios^+$ T cells in the CNS of mice with EAE prior to the loss of pathogenic $CD4^+$ T cells from the CNS and the onset of remission (216). New data show Helios was a marker for peripheral T cells that are being driven to tolerance in response to a genuine autoantigen in autoimmune gastritis (321). Helios was identified as a key inhibitor of *Il2* gene transcription in Treg cells (322); it repressed the *Il2* locus through epigenetic modifications that included histone deacetylation.

Given the importance of pathways downstream of calcitriol signaling to the Th1–Tr1 switch and immune response termination, it is valuable to consider how those pathways could be compromised. CD46 is the receptor for the T lymphotropic human herpesvirus-6; the virions bind CD46 and trigger endocytosis (323). Whether pathogen-mediated CD46 removal would undermine calcitriol action by disabling the Th1–Tr1 switch is not known. Genetically determined defects in IL-10 production and IL-10R signaling would be downstream of a calcitriol-mediated increase in Tr1 cells; both defects were linked to an increased autoimmune disease risk (324–329). The IL-10R signals through tyrosine kinase 2 (TYK2)

and Janus kinase-1 (330). A novel missense mutation in the *TYK2* gene was linked to an increased risk of MS (329). In addition, recombinant hIL-10 did not inhibit proliferation of $CD4^+$ T cells from MS patients; the IL-10R signaling pathway was blocked at the point of STAT3 activation in these cells (267). Defective IL-10R signaling downstream of calcitriol action to induce Tr1 cells could compromise this vitamin D-mediated protective mechanism.

CONCLUSION AND FUTURE RESEARCH DIRECTIONS

This review article sought to summarize and integrate research on vitamin D and $CD4^+$ T-lymphocyte biology in an effort to develop a new understanding of the molecular etiology of autoimmune disease. There is a large, latitude-linked, non-transmissible environmental component that acts at the population level to determine the emergence of an autoimmune phenotype, given an autoimmune disease-susceptible genotype. Diverse and compelling evidence suggests that this environmental component is ambient UVB light exposure catalyzing cutaneous vitamin D_3 formation. Importantly, this environmental component exerts a >100-fold influence on the risk of multiple autoimmune diseases, and on disparate populations with distinct genotypes, dietary habits, and exposures to infectious and commensal organisms, larger than the influence of the strongest autoimmune susceptibility locus (MHCII genes; approximately fivefold) or the infectious organism, EBV (approximately two to threefold).

The strength and universality of ambient UVB light and vitamin D_3 as autoimmune disease risk factors, the vitamin D hormone's established role as a transcriptional regulator of gene expression, and the role of $CD4^+$ T lymphocytes as autoimmune disease initiators and suppressors provided the rationale for closely examining calcitriol regulation of $CD4^+$ T-lymphocyte function in this review. The conclusions supported by experimental evidence are presented in **Box 1**. These conclusions address the Bradford Hill criterion of a plausible biological mechanism that coheres with known biological facts (115). This evidence has contributed to a greater functional and mechanistic understanding of the molecular etiology of autoimmune disease, one of the major challenges in modern immunobiology.

Important questions remain. Regarding paracrine calcitriol signaling to $CD4^+$ T cells, more work is needed *in vivo* to understand the cellular sources of calcitriol, the signals and kinetics that induce and terminate calcitriol synthesis and their relationship to infectious organisms. More work is also needed to understand the signals and kinetics that induce and terminate *VDR* and *CYP24A1* gene expression in specific types of $CD4^+$ T cells *in vivo*, and how gender influences calcitriol responsiveness and calcitriol turnover. Finally, we must learn what factors and mechanisms disrupt paracrine signaling (genetic lesions, drugs, oxidative damage to enzymes, epigenetic dysregulation).

A second and related high priority is probing a possible vitamin D influence on thymic selection. Such an influence would have high significance for our understanding of the molecular etiology of autoimmune disease risk acquisition and our effort to correctly time disease prevention efforts. The data correlating seasonal variation in thymic output inversely with vitamin D status await replication. Assuming reproducibility, mechanistic inquiry will be needed to distinguish potential impacts on thymopoiesis and positive selection from negative selection, and to decipher the calcitriol-regulated genes that influence these processes.

Other questions relate to calcitriol actions on effector $CD4^+$ T cells *in vivo*. Calcitriol suppression of *Il17* transcription and IL-17 protein synthesis has been studied mechanistically in rodents with conflicting results. It will be important to clarify the biochemistry of *Il17* transcriptional and post-transcriptional regulation by calcitriol in Th17 cells *in vivo* in the context of different autoimmune disease settings where physiologically relevant tissue architecture and cell and cytokine microenvironments exist. Other open questions concern the biochemistry of the effector

Box 1 | Summary of main conclusions regarding vitamin D, $CD4^+$ T lymphocytes, and autoimmune disease.

An autoimmune disease phenotype emerges when modifiable environmental stressors act on a disease-susceptible genotype, and exposure to at least one environmental stressor is increasing.

Vitamin D is probably the environmental factor with the greatest influence on the emergence of an autoimmune disease phenotype given a disease-susceptible genotype.

At high latitudes, there is a period of "light starvation" when vitamin D synthesis is negligible; the higher the latitude, the greater is the light starvation period.

Paracrine calcitriol signaling to $CD4^+$ T cells within tissues is likely the major pathway by which sunlight as an environmental factor exerts its influence on the emergence of an autoimmune disease phenotype.

The VDR–RXR complex regulates gene expression in a cell type-specific manner through VDRE in calcitriol-responsive genes.

The hormonal form of vitamin D may influence thymic selection, which would have a significant influence on autoimmune disease risk acquisition and the timing of autoimmune disease prevention initiatives.

The hormonal form of vitamin D may significantly influence autoimmune disease risk and severity by (i) dampening pathogenic Th17 cell IL-17 synthesis, (ii) increasing effector $CD4^+$ T-cell sensitivity to extrinsic cell death signals, (iii) promoting $CD4^+CD25^+FoxP3^+$ Treg cell and $CD4^+IL\text{-}10^+FoxP3^-$ Tr1 cell development and suppressive function, (iv) amplifying a Th1–Tr1 switch that may bridge two apparently stable immune states, an anti-pathogen pro-inflammatory state and a self-tolerant anti-inflammatory state.

During periods of light starvation, phototherapy to increase $FoxP3^+$ Treg cell development may be a particularly efficient method of influencing autoimmune disease risk and severity.

$CD4^+$ T-cell programed cell death pathways that are influenced by calcitriol, and the cellular source and timing of the extrinsic cell death signaling.

Another very high priority is probing how vitamin D promotes $CD4^+FoxP3^+IL\text{-}10^-$ Treg and $CD4^+FoxP3^-IL\text{-}10^+$ Tr1 cell development, stability, and suppressive function. Unanswered questions relate to the biochemical mechanisms for the proposed calcitriol up-regulation of *Ikzf2*, *Il10*, *Ctla4*, and *CD200* gene transcription, and the relationship between *VDR* and *FOXP3*, how each gene may impact the other's expression and the kinetics and stability of the interaction.

Additional questions concern the molecular details of the proposed Th1–Tr1 switch. Whether this switch functions *in vivo* in the context of different autoimmune diseases is a top priority question. Moreover, questions remain regarding the precise biochemical details of the switch, the signals that trigger it, how the signals are transduced to the nucleus, how the gene expression program is altered by those signals, and exactly how calcitriol amplifies the switch. This molecular knowledge is needed to envision and probe how pathogen-mediated engagement of CD46 might compromise the switch. Mathematical modeling of the switch may be helpful to envision interactions between environmental pathogens, the sun-sensitive hormone, the pathogen-specific immune response, immune response termination, and self-tolerance maintenance.

Sir Austin Bradford Hill suggested minimal criteria for judging the causal nature of relationships between environmental factors and disease (115). Many experts believe the experimental evidence linking vitamin D_3 with autoimmune disease risk is sufficiently compelling to satisfy eight of the nine criteria (6). Perhaps, the most important unanswered question derives from Bradford Hill's ninth criterion, experiment. Would an intervention that substantially elevates circulating 25-OHD_3 prevent or change autoimmune disease in light-starved populations with low vitamin D status? Now would be a good time for autoimmune disease researchers to address this unanswered question collaboratively.

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CD4 T cells mediate both positive and negative regulation of the immune response to HIV infection: complex role of T follicular helper cells and regulatory T cells in pathogenesis

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HIV-1 infection results in chronic activation of cells in lymphoid tissue, including T cells, B-cells, and myeloid lineage cells. The resulting characteristic hyperplasia is an amalgam of proliferating host immune cells in the adaptive response, increased concentrations of innate response mediators due to viral and bacterial products, and homeostatic responses to inflammation. While it is generally thought that CD4 T cells are greatly depleted, in fact, two types of CD4 T cells appear to be increased, namely, regulatory T cells (Tregs) and T follicular helper cells (Tfh). These cells have opposing roles, but may both be important in the pathogenic process. Whether Tregs are failing in their role to limit lymphocyte activation is unclear, but there is no doubt now that Tfh are associated with B-cell hyperplasia and increased germinal center activity. Antiretroviral therapy may reduce the lymphocyte activation, but not completely, and therefore, there is a need for interventions that selectively enhance normal CD4 function without exacerbating Tfh, B-cell, or Treg dysfunction.

Keywords: CD4, regulatory T cells, T follicular helper cells, HIV infections, lymphoid tissue

INTRODUCTION

The pathogenesis of CD4 T cell decline during chronic HIV-1 infection is slow and complex. It typically begins with a decrease of CD4 cell counts in peripheral blood, from a median of approximately 800 cells/μl to a median of 500 cells/μl during the first few weeks of primary infection (1–3), but is then followed by a much slower rate of decline over several years (1), eventually leading to opportunistic infections. CD4 cell counts in blood may not accurately reflect cell numbers in secondary lymphoid tissue, since treatment commenced during primary infection leads to a very rapid increase of CD4 cell counts that cannot be accounted for by new production of CD4 T cells and is most likely due to redistribution of resting cells that had been sequestered in lymph nodes (4, 5), as had been suggested for treatment commenced during chronic infection (6–8).

Therefore, depletion within lymphoid tissue early in infection is not so clear. Contradictory results from the SIV model of infection in rhesus macaques suggest either very high levels of infection and loss of CD4 T cells, particularly from gut associated lymphoid tissue (GALT) during primary infection (9, 10), as against sequestration and even proliferation of CD4 T cells in secondary lymphoid tissue during early chronic infection (11–13). In fact, increased rates of proliferation of both CD4 and CD8 T cells are a hallmark of chronic HIV-1 infection (14–16). This increased proliferation begins at the earliest stages of primary HIV-1 infection (5) and is associated with a CD4 response to viral antigens (17). An analogous proliferative response of CD4 and CD8 T cells to vaccinia virus was also clearly observed around day 13 post-inoculation in healthy adults (18). However, in the response to vaccinia virus, as neutralizing antibodies titers increased by day 21 post-inoculation, activation, and proliferation of CD4 and CD8 T cells were rapidly terminated (18), and this was later confirmed using tetramers to identify antigen-specific CD8 T cells (19).

Taken together, these results suggest that changes in CD4 cell numbers during HIV-1 infection are a complex summation of proliferating, but mostly short-lived, CD4 T cells, loss of virally infected cells, changes in trafficking, and feedback regulation to limit responses. These processes will occur in secondary lymphoid tissue and GALT, since they are the major sites of viral replication and antigen deposition (20, 21) and hence antigen presentation. In particular, germinal center (GC) hyperplasia and hypergammaglobulinemia are also absolutely characteristic of established HIV-1 infection (22). For most of the time that HIV-1 infection has been studied, it has been believed that very few antigen-specific CD4 T cells can be found in patients, presumably due to preferential targeting of these cells by virus (23), except that they are somehow protected in rare long-term non-progressors (LTNP) and even rarer elite controllers (EC) [reviewed in Ref. (24)]. Yet, this view of a paucity of HIV-specific CD4 T cells did not take into account the extremely high titers of HIV-specific IgG antibodies found in virtually all patients (22), beginning early in primary infection (25). These antibodies strongly suggest vigorous CD4 T cell help for B-cell responses, consistent with the greatly increased numbers of GCs associated with HIV-1 infection.

This review will discuss the regulatory environment within HIV-1 infected lymphoid tissue, with particular reference to the role of T follicular helper cells (Tfh) in driving B-cell activation and

the role of regulatory T cells (Tregs) in countering lymphocyte activation. Since T cell and B-cell activation and proliferation appear to be unrelenting during early chronic infection, the evidence suggests that the positive regulation by Tfh prevails, and that Treg suppression is insufficient to prevent this.

ANTI-VIRAL CD4 T CELL RESPONSES

CD4 T cell responses are pivotal in the development of effective cellular and humoral immunity against viral infections (26). The crucial role of CD4 T cells was firmly documented in murine models, where adoptive transfer of lymphocytic choriomeningitis virus (LCMV) specific CD4 T cells into mice with chronic infection restored function to exhausted CD8+ T cells and reduced viral burden (27, 28). Similarly in human cytomegalovirus (CMV) infection, loss of CD4 T cell function correlated with end-organ disease, and adoptive transfer of CMV-specific CD4 T cells into infected patients leads to reduction in viremia and immune restoration (29, 30). In the case of HIV-1 infection, LTNP and EC may control viral replication with the help of cytotoxic CD4 T lymphocytes specific for p24 (31, 32), and characteristically have CD4 T cells that vigorously proliferate in response to HIV-1 antigens, compared to non-proliferative CD4 T cells from subjects with progressive disease (24).

While exhaustion and dysfunction of anti-viral effector T cells have been suggested as a major factor in chronic viral infections, particularly the LCMV model in mice (33), the role of neutralizing antibodies, generated through CD4 help for B-cells in GCs may well be the ultimate determinant of outcome (34, 35). Recently, it has been reported in the LCMV model that late development of a neutralizing antibody response correlates with eventual clearance of the chronic infection, rather than T cell immunity (36). This clearance is associated with a slow development of viral antigen-specific Tfh (37). At the same time, a negative role for Tregs in anti-microbial responses in animal models, and outcome of these infections, is clearly established (38), and are highly likely to provide negative feedback regulation to limit tissue damage. Therefore, there are diverse effector and regulatory roles of CD4 T cells in the anti-viral response.

Human immunodeficiency virus (HIV) infection is a prime example of the clinical relevance of CD4 T cell loss, where progressive depletion of the T helper (Th) population leads to increased morbidity and eventual mortality if untreated. Depletion of CD4 T cells is believed to be mostly due to direct infection of this subset (21). However, loss of cells may also be due to chronic immune activation, secondary to chronic exposure to microbial products translocated across epithelial barriers depleted of CD4 T cells during primary HIV-1 infection (39) and alteration of homeostasis due to eventual fibrotic changes to lymphoid tissue (40). Direct anti-viral effector functions of human CD4 T cells are quite clear, particularly cytotoxic activity in HIV-1 and CMV infections, respectively (31, 32, 41, 42). However, the demonstrated cardinal role of the various subsets of CD4 T cells in experimental models of immune responses is to ensure optimal help to other lymphocytes, especially B-cells (Tfh subset of CD4 T cells) and CD8 T cells, as well as to recruit monocytes (Th1), eosinophils and basophils (Th2), and neutrophils (Th17), and also to limit responses (Tregs) (43).

Treg PHENOTYPES AND MECHANISMS OF ACTION

Regulatory T cell-mediated suppression of inflammation serves as a crucial mechanism in the prevention of autoimmune disorders and the control of negative regulation of inflammatory diseases. Tregs are indispensible for the maintenance of homeostasis of the immune system that limits the magnitude of effector responses and allows the establishment of immunological tolerance. Two main types of Tregs have been identified, they include natural (or thymic) and induced (or peripheral) Tregs and both play important roles in turning down immune responses (44, 45).

Naturally arising CD4 regulatory T cells (nTregs) develop in the thymus and are primarily engaged in dominant control of self-reactive T cells (46). The initial evidence in support of thymic generation of cells that can mediate immune tolerance through suppression of other cells materialized from studies of neonatal thymectomy (47), but differentiation of inducible Treg cells occurs in the periphery, mainly within lymphoid tissue including GALT (48), where peripheral Tregs have increased affinity to non-self Ags, e.g., allergens, food, and commensal micro-biota. IL-10 producing regulatory T cells, termed Tr1 cells, are another subset of CD4 T cells, which produce the anti-inflammatory cytokines IL-10 (49) and transforming growth factor-β (TGF-β), and are involved in down regulating immune responses toward allergens and various antigens such as nickel and insect venom, as well as controlling autoimmunity, and preventing allograft rejection and graft versus host disease (GvHD) (50).

The transcription factor Foxp3 has been identified as the master regulator of Treg differentiation (45). In humans, CD25 alone cannot distinguish Tregs from activated CD4 T cells, and staining for Foxp3 involved fixation and permeabilization, thus it was necessary to find an additional marker for the identification of Tregs. It was discovered in 2006 by Seddiki et al. that the IL7Rα (CD127) is expressed at low to intermediate levels on the surface of Tregs and the combination of CD25+ CD127lo can be used to distinguish Treg from other CD4 subsets; CD25+ CD127low Tregs contain high amounts of Foxp3 and can suppress immune responses *in vitro* (51, 52).

Tregs IN HIV INFECTION

Regulatory T cells have been associated with several roles in HIV infection, which may occur at different times during the infection process and may be affected by ongoing therapy. The negative roles of Tregs in HIV infection include inhibitory effects on effector T cells during early infection (53); may serve as possible targets for HIV replication (54); and may have the ability to suppress HIV-specific responses that can lead to inhibition of T cell responses to HIV and increase viral persistence, leading to immune exhaustion (55, 56). Possible beneficial roles of Tregs may be their ability to reduce immune activation (57–59), particularly in situations of increased lipopolysaccharide (LPS) concentrations (60), and this restriction of activation of CD4 T cells could limit their loss.

A subset of Tregs can express CCR5, at a level comparable to other conventional CD4 T cells (Zaunders et al. unpublished data), which makes them susceptible to HIV infection (61–63). Naïve Tregs (nTregs) are able to upregulate CCR5 and CXCR4 following TCR stimulation, and when compared to conventional effector T helper cells, Tregs are less susceptible to HIV R5 strain

but more susceptible to X4 strain *in vitro* (61). However, it is doubtful whether Tregs are major targets of HIV *in vivo* due to the small absolute number of CCR5+ Tregs [approximately 20 cells/μl in peripheral blood; (Zaunders et al. unpublished data)], and the relatively small amount of HIV DNA found in Tregs from HIV+ subjects reflects this (63). Rather the majority of Tregs may serve a role in inhibiting viral replication in other target CD4 T cells during early infection, which may assist in preventing the initial spread of the virus from the mucosal sites to lymph nodes (64, 65).

Despite evidence of some Tregs being infected, their suppressive function is largely retained in chronic progressive HIV-infection, originally shown through depletion experiments (53, 55, 57, 66), but more recently through analysis of the function of purified Tregs (67, 68). However, in one study of a small number of HIV+ subjects with immune reconstitution disease following antiretroviral therapy (ART), Tregs exhibited reduced suppression, and at the same time, responder cells from the same patients were less able to be suppressed by Tregs from healthy controls, suggesting overall impairment of Treg suppression (69).

During chronic HIV infection, the absolute Treg numbers in peripheral blood declined, but the proportion of Tregs among CD4 T cells is increased, regardless of the phenotype that was used (54, 70). This suggests that there is relative resistance of Tregs to the cell-depleting effects of HIV, compared to other CD4 T cell subsets. In one study, there was a relatively low proportion of Tregs in HIV+ EC that correlated with slightly higher T cell activation (71), but in an earlier study, no such difference had been found (18, 72). Other studies have shown that absolute numbers of Tregs in LTNP was similar to progressors, but frequencies were much lower than uninfected controls (62, 67, 73).

Accumulation of Tregs relative to conventional CD4 T cells during HIV infection could be explained by several mechanisms, which may include an increase in the proportion of CD25+ FoxP3+ cells regressing the thymus in HIV-infected individuals (74–76). Second, preferential survival and proliferation of Tregs may result from decreased sensitivity to TCR re-stimulation compared to non-Tregs, and a substantial resistance to activation-induced cell death (77). It has also been shown that exposure of Tregs to HIV-gp120 promoted their survival via a cAMP dependent pathway (78), inhibited Treg apoptosis via up-regulation of the anti-apoptotic protein Bcl-2 (79), as well as accumulation of Tregs in peripheral and lymphoid tissues (80). Furthermore, there is an increase in Ki67 (a cell cycle marker) expression in circulating Tregs from untreated, chronically infected patients prior to undergoing ART (81, 82). Third, there may be increased conversion of peripheral naïve CD4 T cells into induced Treg phenotypes. Plasmacytoid dendritic cells (pDCs) represent a small proportion of dendritic cells (DCs) (0.2–0.8% of PBMCs) (83) that have been identified as the main subset of DCs that have the ability to convert allogeneic non-Tregs into CD25+ FoxP3+ Tregs, when exposed to HIV (84). Several studies have shown positive correlations between pDCs and Treg percentages post-therapy (83, 85, Phetsouphanh et al. unpublished data), and indicates that pDCs may play a role in the genesis of peripheral Tregs. Possible reasons for this include (a) development of semi-mature mDCs through HIV interaction that leads to stimulation and proliferation of Tregs (86), which also occurs in SIV infection (87); (b) HIV-stimulated pDCs could induce Treg proliferation by producing indoleamine-2,3-dioxygenase (IDO), and Tregs induced by pDCs have been shown to inhibit maturation of bystander conventional mDCs (84, 88).

ROLE OF CD39 AND DISEASE PROGRESSION DURING HIV-1 INFECTION

Two ectoenzymes: CD39 [ecto-nucleoside triphosphate diphosphohydrolase (E-NTPDase)] and CD73 [5′-nucleotidase (5′-NT)] involved in catabolism of extracellular adenosine triphosphate (ATP) have recently been shown to be highly expressed on Tregs in mice, whereas, in humans only CD39 is present and is highly enriched in antigen-specific Tregs (89–91). High levels of extracellular ATP indicate tissue destruction and inflammation. The presence of extracellular ATP can be sensed by purinergic receptors. CD39 can hydrolyze ATP or adenosine diphosphate (ADP) to adenosine monophosphate (AMP) and CD73 can further catabolize AMP to adenosine. Removal of extracellular ATP by CD39 may allow Tregs to migrate to inflamed sites and permit Treg cells to quench ATP-driven pro-inflammatory processes in multiple cell types, in particular, ATP-driven DC maturation. The immunomodulatory effects of ATP removal by CD39 is further enhanced by the generation of adenosine, which binds to A2A adenosine receptor (A2AR) and elicits inhibitory functions of DCs as well as activated T cells (62, 92). This mechanism is widely believed to be important in the observed immunological tolerance of tumors (93).

A consistent feature of Tregs in HIV infections is that they express high levels of CD39, and this high level remains unaltered even with therapeutic interventions (62, 82). Elevated CD39+ Treg frequencies positively correlate with plasma viral load and negatively with CD4 recovery (94, 95). Nikolova et al. demonstrated that a genetic variant of the CD39 gene *ENTPD1* (ecto-nucleoside triphosphate diphosphohydrolase 1) was associated with lower expressions of the CD39 protein, and this led to a slower progression to AIDS (95). High frequencies of CD39+ HIV-specific Tregs were identified in HIV-infected individuals pre-treatment, and low frequencies of CD39− HIV-specific non-Tregs were associated with higher viral load (91). Additionally, blocking of CD39 via monoclonal antibodies eliminated Treg-mediated suppression of CD8+ cytokine production when stimulated with Gag (95). Taking together, CD39+ Tregs may be critical for the inhibition of T-cell associated immune responses, and may control HIV-induced T cell activation, which may reduce HIV replication (91, 96).

Overall, then HIV-1 infection is associated in general with a modest increase in Tregs relative to the conventional CD4 T cells that they normally regulate, and, if anything, may be more active than normal.

T FOLLICULAR HELPER CELLS AND MECHANISMS OF ACTION

T follicular helper cells provide help to B-cells in GCs of secondary lymphoid organs and are crucial for GC formation, immunoglobulin class-switch recombination, somatic hyper-mutation, and differentiation of B-cells into long-lived memory B-cells and plasma cells (97). Tfh cells are central to the generation of efficient neutralizing and non-neutralizing antibody responses in HIV infection and will be essential in generating an effective vaccine (98, 99).

T follicular helper cells express high levels of surface markers program death-1 (PD-1) and chemokine CXC receptor 5 (CXCR5), which make them phenotypically distinct from other T helper cell lineages and from peripheral CXCR5+ cells with helper activity for B-cells *in vitro* (as discussed below) (97, 100). However, Tfh cells' identity as a separate lineage of T helper cells was established when B-cell lymphoma 6 (Bcl-6) was discovered to be necessary and sufficient to drive their differentiation (101, 102).

Naïve CD4 T cells' multi-step differentiation toward Tfh cells begins with antigen-presenting DCs in the T cell zone (103), stimulating Tfh through TCR, and costimulatory CD28 and ICOS (104). Secretion of IL-6 by DCs serves as a primary signal for the induction of Bcl-6 expression in CD4 T cells in a STAT3-dependent manner, which subsequently drives the expression of Tfh cell signature genes critical for T cell: B-cell interaction, including *Cxcr5*, *Icos*, *Pdcd1*, *Sh2d1a*, and *Cd40l* (105). Another DC secreted cytokine IL-27 induces the expression of transcription factor c-Maf, which cooperates with Bcl6 to enhance the expression of the above Tfh associated genes and induces IL-21 production (106). IL-21 acts to promote Tfh cell differentiation and maintain Tfh cells, probably directly, as well as via its role in inducing Bcl-6 expression and differentiation of GC B-cells, which in turn reinforce Tfh differentiation (107–112). During this process, IL-21 can also induce the expression of B lymphocyte-induced maturation protein 1 (Blimp-1), which is required for the switch from GC B-cells to plasma cells, and activation-induced cytidine deaminase (AID), which is required for class switched recombination (CSR) (112, 113).

T follicular helper differentiation and activity may be regulated at several levels. OX40 (CD134) signaling promotes expression of transcription IRF4 that may cooperate with Bcl6 to maintain Tfh cells (114, 115). High levels of PD-1 on Tfh cells binding to PD-L1 on B-cells provides inhibitory signal to Tfh (116–118). IL-2 signaling prevents Tfh cell differentiation by activating STAT5, which subsequently induces Blimp-1, which represses Bcl6 (119, 120), whereas signaling by interferons or IL-12 may induce T-bet, which complexes with Bcl6 to preemptively repress Blimp-1 (105, 121). Tfh cell differentiation is also reportedly suppressed by CD8+ regulatory cells (122), plasma cells (123), but positively regulated by available antigen presentation (103, 124).

FOLLICULAR Tregs

Follicular Tregs (Tfr) cells were first described as a subset of Tregs that derive from Foxp3+ thymic Tregs and directly repress Tfh cell proliferation and numbers in the GC (111, 125, 126). Tfr and Tfh cells share differentiation and regulation mechanisms, including up-regulation of Bcl6, which instructs the expression of CXCR5, PD-1, and ICOS, and requires CD28 and SAP signaling, as in Tfh cells (111, 125). PD-1/PD-L1 signaling negatively regulates Tfr cells, not only their expansion but also their suppressive ability (127), although the actual number of Tfr in lymph nodes is very small relative to Tfh, in either non-human primate or human lymph nodes (Xu et al. unpublished data). Circulating Tfr, CXCR5+ICOS+Foxp3+ CD4 T cells, have also been described (127). However, whether these cells in peripheral blood have truly come out of a GC reaction and whether they will migrate back to the GC upon recall stimulation needs to be further investigated, to classify them as a distinct Treg subset.

CIRCULATING Tfh-LIKE CELLS

A subset of circulating memory CD4 T cells bearing the phenotype of CXCR5+, and more stringently CCR7lo, PD-1+, and ICOS+, have been termed "circulating Tfh," "blood Tfh," "peripheral Tfh," or "memory Tfh" cells and are now being intensively studied (128). This reflects the need for surrogate biomarkers in the periphery to correlate with the number of *bona fide* Tfh cells in lymphoid tissue (129, 130). Whether circulating CXCR5+ Tfh-like cells truly represent the memory form of Tfh cells is controversial, although most current evidence suggests that is the case.

First, CXCR5 and PD-1 are stably expressed on these cells rather than a transient response to activation (131). Second, at least a subpopulation of blood CXCR5+ CD4 T cells are highly functional in helping B-cells to survive, to differentiate into plasmablasts, and to produce class switched antibodies upon stimulation *in vitro* or in response to vaccination *in vivo* and this B-cell help is mediated by up-regulation of CD40L or ICOS, and secretion of large amount of IL-21 (130–132). Third, it has been demonstrated in mice that blood CXCR5+ CD4 T cell differentiation is dependent on Bcl6 and ICOS, but not SAP, suggesting that circulating CXCR5+ CD4 T cells are precursors of GC Tfh cells (128). Finally, it has been demonstrated in mice that Tfh cells could revert to memory cells in the absence of antigen and could differentiate into conventional effector cells or Tfh cells upon recall (133, 134). However, blood c-Tfh-like cells and Tfh cells in lymphoid tissue are clearly phenotypically different, particularly with respect to expression of PD-1 and Bcl6 *ex vivo* (135, 136). Recent RNA sequence data also showed that a subset of blood CXCR5+, with the highest helper activity for B-cells *in vitro*, exhibited a gene expression profile more closely related to non-Tfh CD4 T cells than Tfh cells in tonsil (100). Further investigation is required to harmonize the observations and understand the relationship between Tfh cells in lymphoid tissue and different subsets of blood CXCR5+ CD4 T cells.

Tfh IN HIV INFECTION

In recent years, Tfh cells have been studied intensively in the context of acquired immunodeficiency such as SIV/HIV infection. Early in the 1990s, HIV-1/SIV RNAs had been detected by *in situ* hybridization at high concentrations in the lymph node GCs (20, 21, 137–139). Follicular dendritic cells (FDCs) have been recognized as a major reservoir for virus in lymphoid tissues, facilitating infection of CD4 T cells (140, 141). However, direct evidence for Tfh cells harboring HIV/SIV DNA was only available in the last 2 years (135, 142, 143). Small numbers of Tfh cells were found to be productively infected (135) and replication competent virus could be isolated from infected Tfh cells (143), indicating that Tfh cells are not only a major target of HIV/SIV infection but also a significant CD4 compartment for viral replication and production. This was paradoxical as Tfh cells express very low levels of CCR5 and other HIV/SIV entry coreceptors (135), but they were infected with HIV/SIV at higher or comparable levels, even at a very early stage of infection (135, 143).

More surprisingly, despite being infected with the virus, both cell number and relative percentage of Tfh cells increased during

the chronic phase of HIV or SIV infection (135, 142–145). The frequency of Tfh cells correlated with plasma viral load, which suggests that Tfh may be a source of circulating virus (145). The expansion of Tfh cells also correlates positively with the frequency of GC B-cells and antibody production (143, 145). Aberrant Tfh cell expansion is associated with B-cell abnormalities such as polyclonal B-cell activation (146), hypergammaglobulinemia (142, 147), and B-cell driven lymphadenopathy (99, 148, 149). In contrast, broadly neutralizing antibodies (bNAbs) specific for HIV occur very rarely in natural infection (150). bNAbs exhibit unusually high levels of affinity maturation, a result of somatic hypermutation (151). Although this was thought to be a by-product of persistent infection, an optimal GC reaction may be required for B-cells to undergo multiple rounds of mutation and selection.

The underlying mechanisms that lead to abnormal GC B-cell responses and antibody production caused by Tfh cell expansion are not fully understood, but at least one mechanism has been proposed. During HIV infection, PD-L1 levels on GC B-cells, but not in memory B-cells, were elevated. Increased PD-1/PD-L1 signaling between Tfh and GC B-cells results in reduction of ICOS expression, which in turn affects downstream IL-21 secretion (118). Since IL-21 is required for GC B-cells survival and differentiation into long-lived plasma cells, GC B-cells receiving inadequate help from Tfh cells may fail to function optimally.

cTfh-like cells, irrespective of how they were defined, were reported to decrease in treatment naïve HIV+ patients (100, 152). This might be a result of CXCR5 internalization in response to elevated serum CXCL13 levels in untreated patients (100) and ART was able to normalize the frequency of cTfh-like cells (100). Such observations are in contrast to the majority of scenarios in primary immunodeficiencies and autoimmune diseases, where blood CXCR5+ CD4 T cell frequencies decrease or increase along with Tfh cells in lymphoid tissue (129, 153). This observation indicates that, if the circulating CXCR5+ CD4 T cells are indeed the memory form of Tfh cells, or traffic out of lymphoid tissue in autoimmune conditions, HIV infection may alter the pattern of Tfh cell trafficking.

The ability of cTfh-like cells' to help B-cells *in vitro* is compromised, in at least a proportion of HIV+ patients (100, 152). In one report, PD-1+CXCR3−CXCR5+ CD4 T cells in peripheral blood positively correlated with bNAb development in HIV+ donors (131), whereas in another report no such correlation was found (100). This discrepancy likely arises from differences in patient samples and cell subsets studied.

Treg AND Tfh IN TISSUES

An accumulation of Tregs in gut mucosa and lymphoid tissues has been reported in HIV infection (64, 154). Tregs express the lymph node homing marker CD62L (155, 156) and gut homing integrins α4β7 (157), although the proportion of α4β7+ cells is relatively low, typically around 10% of Tregs (Zaunders et al. unpublished data). The expression of these receptors increases in Tregs following HIV-1 exposure *in vitro* (80). This may explain the accumulation of Tregs in lymphoid and mucosal tissues, where Treg frequencies are much higher than peripheral blood (62, Xu et al. unpublished data). Characteristic molecules such as FoxP3, cytotoxic T lymphocyte antigen 4 (CTLA-4), glucocorticoid-induced-TNFR family related receptor (GITR), and CD25, have been shown to be overexpressed on Tregs in tonsil and lamina propria of duodenal mucosa of untreated patients compared to treated (64, 154).

Other functional Treg markers such as IDO, TGF-β, and CD80 were also markedly increased in tonsillar tissue of untreated patients (154). Furthermore, the prevalence of Treg correlated better with viral load in tissues compared to plasma viremia (158). GALT also represents a major site of HIV replication and CD4 T cell depletion (96, 159, 160). HIV infection leads to a loss in Th17 cells that are vital for mucosal immunity against other pathogens, which may play a role in the increased microbial translocation across the gastrointestinal mucosa leading to systemic immune activation (161, 162). A relative increase in Tregs may play a role in aggravating this effect by inhibiting HIV-specific immune responses in the GALT (163, 164).

There is evidence that Tregs can enter GCs *in vivo*, and suppress CD4 T cell help for B-cells *in vitro* (165) and also directly suppress B-cells (166). The reported mechanism required cell contact, consistent with up-regulation of CXCR5 on Tregs activated *in vitro* and chemotaxis directed by CXCL13. Furthermore, in one study, it was shown that Treg suppression of GC reactions *in vivo* could be counteracted by treatment of mice with antibodies to GITR, TGF-β, or anti-IL-10 (167). As detailed above, there has now been described a small subset of follicular CD4 T regulatory cells, Tfr, which express both Bcl-6 and Foxp3 and exhibit suppressive activity (111, 125, 126). However, these cells appear to be generated during the course of a GC reaction (134), and also may enter the circulation as long-lived memory cells (127, 134). Therefore, these cells may represent a potent feedback mechanism, but it is unclear whether they would normally regulate the conditions during HIV or SIV infection that drive lymph node hyperplasia. Generalized T cell activation during HIV or SIV infection occurs in the T cell areas and regulation of the initial CD4 activation, prior to expression of Bcl-6 and CXCR5, is more likely to be mediated by canonical Tregs.

It has been reported separately that Tregs and Tfh are both increased in lymph nodes in HIV or SIV infection, with the latter possibly showing greater increases, as detailed above, but direct quantitation of both subsets within the same tissue during HIV infection has not been documented. Xu et al. recently studied T cells from lymphoid tissue using fine needle aspiration (FNA) (135) in pigtail macaques. This technique has now been applied to lymph nodes in HIV-infected and uninfected human subjects and it was confirmed that the ratio of Treg to Tfh was <2:1 in lymph node cells from HIV-infected subjects, but was 30:1 in uninfected subjects (Xu et al. unpublished data).

An important consideration is how the increase in Tfh is maintained over such long periods of time, probably years. The lifespan of individual Tfh cells is unknown, although, where studied, they exhibit low levels of Ki67 and are generally not prone to spontaneous apoptosis (142, Xu and Zaunders, unpublished data). Similarly the lifespan of individual GCs is not clear, since they begin to regress by day 14 after primary vaccination in a mouse model and do not greatly increase again with secondary challenge (168), although some studies have reported that GCs can be long lasting in mice, possibly up to 180 days (169, 170). One possibility for long-term elevation of Tfh cell numbers in HIV-1 infection

could be due to their own success, if they help generate antibodies that put pressure on the virus, which causes mutations in gp120, which in turn generates neo-antigens, and which in turn generate further immune responses. A striking feature of HIV-1 infection is the continual generation of envelope variants within each patient (171), and later it was found that neutralizing antibody responses were associated with sequential escape mutations (172). Therefore, much more work is required to understand the direct interactions between Tfh and Tregs, how GCs normally regress at the end of an immune response and why this does not happen in HIV-1 infection. Also, the lack of a strictly parallel increase of Tregs and Tfh cells in lymph nodes may indicate that other factors such as cytokines or transcription factors can impact separately on the dynamics of Treg and Tfh in HIV infection (**Figure 1**).

ROLE OF CYTOKINES

IL-6 is a pleiotropic cytokine produced by myeloid cells (monocytes, macrophages, and DCs) (173, 174). It binds to a receptor complex consisting of soluble/transmembrane IL-6 receptor (IL-6R) and the signal-transducing receptor subunit gp130, binding of the receptor potently activates signal transducers and activators of transcription 3 (STAT3), and to a minor extent STAT1 (175, 176). Plasma IL-6 was found to be elevated in HIV infected patients (177) and SIV-infected macaques, but not in SIV-infected African green monkeys, the natural host of SIV (142, 178). ART reduced plasma IL-6 levels, but this reduction never reached levels seen in uninfected donors (179). IL-6 levels in lymph nodes, in contrast, seem to be high in both uninfected and HIV infected samples (174, 179), although it was reported that IL-6 mRNA levels

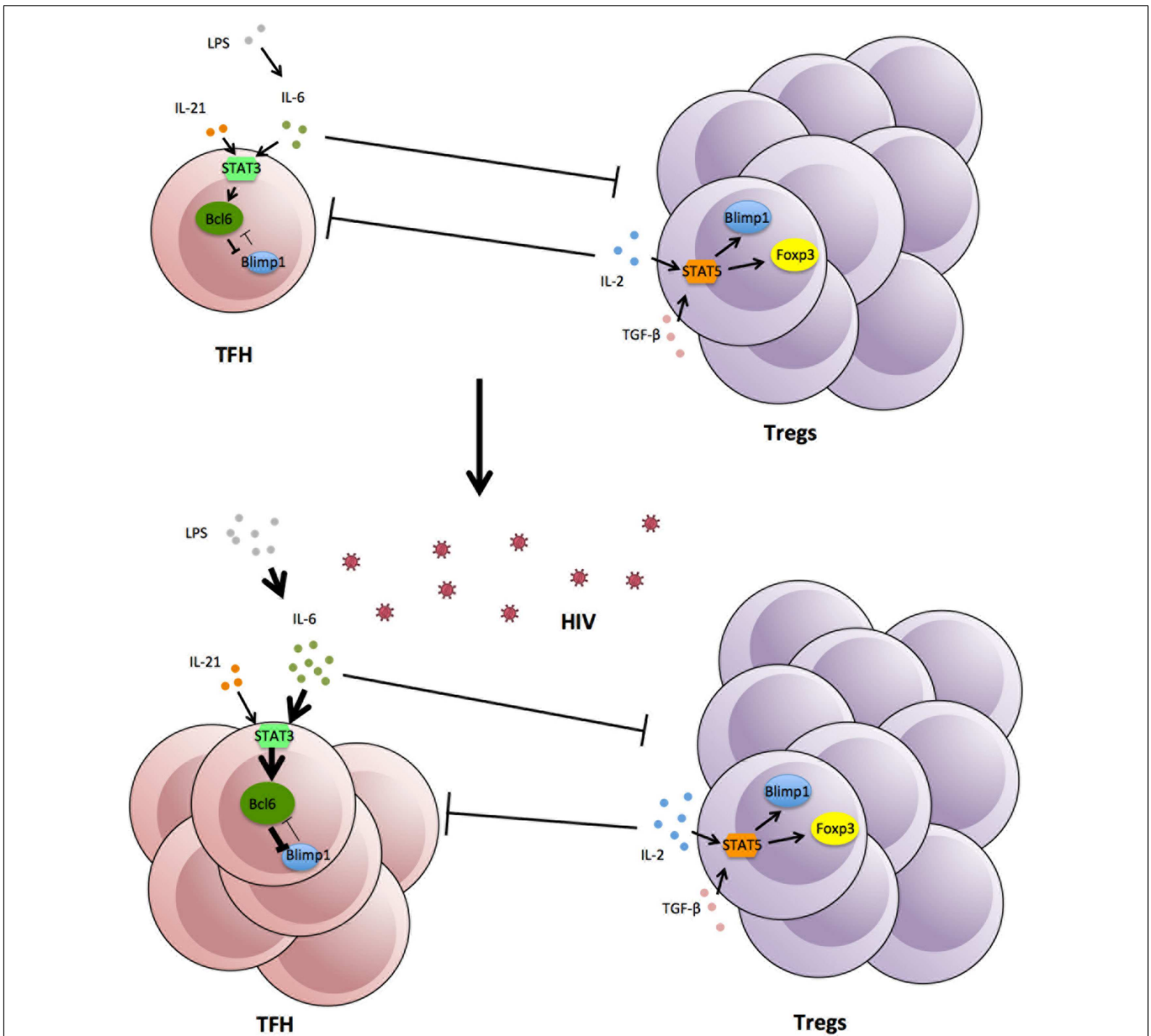

FIGURE 1 | Expansion of Tfh in lymphoid tissue following HIV-1 infection, associated with increases in cytokines and viral and bacterial products.

were increased in lymph nodes from macaques as early as 7 days post SIV infection (180). However, HIV itself does not seem to be the direct driver of IL-6 production (179, 181, 182). Instead, LPS alone markedly induced IL-6 production at low concentrations (181, 182). Increased plasma LPS is not only a property of pathogenic SIV infection but has also been reported in progressive HIV infection (159). Again, ART reduced plasma LPS level significantly but failed to reach levels found in uninfected donors (159). Therefore, persistently high levels of LPS despite ART may result in persistently high levels of IL-6, and subsequently Tfh cell accumulation in chronic HIV/SIV infection (183).

Transforming growth factor-β is a binary cytokine in CD4 T cell induction. Together with IL-2, it stimulates the differentiation of peripheral Tregs via STAT5; with IL-6, it inhibits the generation of peripheral Tregs but induces the development of Th17 cells via STAT3 (184, 185). The reciprocal relationship between Th17 cells and Tregs has been well documented (184, 186). However, the relationship between Tfh cells and Tregs remains elusive. Oestrich et al. showed that in IL-2 limiting conditions Th1 cells can upregulate BCL-6, which converts these cells into Tfh-like cells with similar gene profile including up-regulation of CXCR5 (121). High levels of exogenous IL-2 have been reported in HIV infected subjects with high viral load (187, 188). As Tregs are known to mop up IL-2 for homeostatic proliferation, this may explain both the accumulation of Tregs and expansion of Tfh in tissue. Tsuji et al. reported on the generation of Tfh cells from Foxp3+ Tregs in gut Peyer's patches, but not in spleen or lymph nodes (189). However, more work is required to confirm this finding.

Regulatory T cells and Tfh share an extremely important property, namely, low expression of the IL-7 receptor alpha chain, CD127 (51, 135), which distinguishes them homeostatically from the vast majority of CD4 T cells. This feature may be highly relevant to their ascendency during chronic HIV-1 infection if damage to lymph nodes (40) affects IL-7 signaling.

ROLE OF TRANSCRIPTION FACTORS

Bcl-6 and BLIMP-1 are key antagonistic transcriptional regulators of effector and memory differentiation in CD8+ and CD4 T cells, but were first identified as critical regulators of B-cell maturation and memory formation, determining cell fate decisions (101, 190). Bcl-6 and BLIMP-1 have been studied in HIV infection, and BLIMP-1 is highly expressed at both the mRNA and protein levels in CD4 T cells in patients with chronic HIV infection compared to LTNP (191). The lower expression of BLIMP-1 in CD4 T cells from LTNP correlates with lower levels of exhaustion in CD4 T cells found in LTNP (191). The expression of BLIMP-1 can be modulated at the translational level by microRNA-mir9 and Seddiki et al. demonstrated that BLIMP-1 levels decreased following treatment with pre-mir-9, while IL-2 expression was increased. Levels of mir-9 were also found to be elevated in LTNP compared to chronically infected subjects (191, 192). BLIMP-1 has also been found to be required for effector Treg differentiation and is essential for IL-10 production (193). Therefore, the level of BLIMP-1 expression in Tregs in chronically infected subjects and LTNP should be investigated to further delineate the importance of this transcription factor in HIV infection. Also, the antagonistic effects of Bcl-6 and BLIMP-1 may present a therapeutic target for the manipulation of T helper subset fate decision.

Tregs/Tfh AS POTENTIAL TARGETS OF HIV IMMUNOTHERAPY

As Tregs and Tfh cells play crucial roles in homeostatic immune responses and the dysregulation of these cells due to HIV-infection causes severe morbidity, therefore Treg and Tfh cells are of interest as potential targets for immunotherapeutic intervention. Many strategies have been implemented to influence the frequency and function of these cells, such as inhibition of specific enzymes, monoclonal antibody (mAb) therapy, and cytokine based clinical trials, as detailed below.

The enzymatic activity of IDO has the ability to influence the Th17/Treg balance, and can enhance the suppressive activity of Tregs. Thus, modulation of IDO in disease is of therapeutic interest. In an animal model of HIV-1 encephalitis, inhibition of IDO via 1-methyl-D-tryptophanh (1-MT) enhances the generation of HIV-specific cytotoxic T cells, which led to the destruction of macrophages in the brain (194). In other observations, IDO seems to synergize with therapy to control viral replication in lymph nodes and plasma of macaques infected with SIV (195). The inhibition of both IDO and CTLA4 in combination has been shown to transiently reduce the kynurenine/tryptophan ratio, increase Th1 proliferation and block Treg suppressive functions. A side effect of this combination therapy, however, resulted in fulminant diabetes with severe infiltration of lymphocytes in the pancreas (196). Taking these previous findings into consideration, potential IDO inhibitors need to be studied intensively in the context of HIV therapy.

Program death-1 is an important marker that modulates the inhibitory pathway, which regulates the T-cell receptor signaling (197). This has been studied intensively in chronic viral infections (198–201). PD-1 is expressed at high levels on HIV-specific T cells during HIV infection, and correlates with plasma viral load, reduced cytokine production, and impedes proliferation of HIV-specific CD8+ T cells (202). PD-1 blockade enhanced the capacity of HIV-specific CD8+ T cells to survive and proliferate during infection, as well as intensifying HIV-specific CD8+ T cells responses (202). PD-1/PD-ligand axis enables the conversion of Th1 cells into Tregs, thus by blocking PD-1 with a mAb may aid the initial response to HIV in early infection (203). Consistent with a role for PD-1/PD-L1 and PD-L2 in Tfh function (116), it has been shown that PD-1 blockage on PD-1 high Tfh cells co-cultured with B-cells significantly inhibits IgG production (204). As Tfh cells accumulate in HIV infection and these cells predispose to B-cell related morbidities, PD-1 blockade could be considered as potential therapeutic intervention.

Cytotoxic T lymphocyte antigen 4 (CD152) is another target for therapeutic intervention. The administration of anti-CTLA-4 blocking antibodies was not detrimental and had beneficial virological effects in SIV-infected ART treated macaques. Decreases in IDO, TGF-β, and viral RNA expression in tissues were observed (205). However, in untreated SIV infection, CTLA-4 inhibition did not restore SIV-specific immune responses and there was an increase in viral replication and CD4 depletion, particularly at mucosal sites (206). It was found that even at the earliest stages of

primary HIV-1 infection, Gag-specific CD4 T cells were dominated by expression of CLTA-4 (18), and it was found that *in vitro* blockade of CTLA-4 significantly increased CD4 T cell proliferation and improved cytokine secretion from HIV-specific CD4 T cells responding to cognate antigen (207). It has also been shown that combination blockade of PD-1 and CTLA-4 reduced Treg activity in cancer (208). However, whether the same approach in HIV infection would yield similar results, remains to be ascertained.

Cytokine based clinical trials have been implemented in the past to facilitate the restoration of T cells in HIV infection. IL-2 is a critical cytokine needed as a strong stimulatory signal for Treg development and function (209, 210). IL-2 was the first candidate cytokine used as an immunotherapeutic agent to boost total CD4 cell counts, although one of the benchmarks of treatment was an increase of CD4+CD25+ T cells, which potentially included Treg cells. Two major phase III clinical trials were conducted, but despite substantial increases in CD4 T cell count, IL-2 in addition to ART yielded no clinical benefit compared to ART alone, in either study (211). These trials showed predominant increases in CD4+CD25+CD127lowFoxP3+ cells, and these cells exhibited molecular and suppressive functions such as those found in Tregs (75). However, there was also a lack of protective effect of IL-2 expanded CD4 T cells on HIV disease progression. In addition, there were potential deleterious effects observed in treated patients relating to cardiovascular and inflammatory events (212). A possible explanation for this is the expansion of suppressive Tregs with truncated STAT5 expression, rendering these IL-2 expanded cells ineffective in protecting against disease progression (96, 212). Thus, other trials using other immunological-based compounds must carefully monitor the phenotype and function of the expanded CD4 T cells.

IL-7 immunotherapy was also developed for HIV infection, first conducted in animal models, where increases in CD4 T cell counts were observed in the absence of immune activation (213, 214). Contrary to IL-2 based immunotherapy, administration of IL-7 resulted in the expansion of CD4 T cells without increasing the frequency of immune-suppressive Tregs, consistent with the low levels of the IL-7 receptor (CD127) expressed on Tregs (51). Also, in one study, *in vitro* incubation in the presence of IL-7 reduced the suppressive activity of Tregs isolated from HIV+ subjects (69), suggesting that IL-7 therapy may have another effect to further boost conventional T cell responses. Due to these differences in responsiveness to IL-7, immunomodulation using various strategies involving either blocking of the receptor to suppress responses or addition of IL-7 to boost responses is currently being investigated in a number of other clinical situations, including autoimmunity, cancer vaccines, and transplant tolerance [reviewed in Ref. (215)].

IL-21 is a pleiotropic cytokine that is important for T cell and B-cell proliferation and maintenance (216) and is produced most abundantly by Th17, Tfh, and natural killer T (NKT) cells. As discussed above, Tfh cells require this cytokine to enhance proliferation and function. Previous animal models have also shown that IL-21 had stimulatory effects on NK cells and CD8+ T cells, and this effect leads to anti-tumor activity (217). Now, IL-21 has been used in phase I and II trials in cancer and early results demonstrated that recombinant IL-21 administration has an acceptable safety profile and has demonstrated encouraging activity in early phase renal cell carcinoma and melanoma trials (218). This makes IL-21 a potential agent for Treg/Tfh modulation, as IL-21 has inhibitory effects on Treg differentiation via the reduction of IL-2 production from other CD4 T cells (219). Since Tfh cells require IL-21 for homeostatic proliferation and are suited to function in low IL-2 conditions, strategies to modulate IL-21 signaling could be used to modulate Treg/Tfh dynamics in HIV infection.

CONCLUSION

HIV-1 infection leads to chronic activation of T cells, B-cells, and myeloid lineage cells within lymphoid tissue, as a result of the combined effects of the host immune response, the increased presence of viral and bacterial products that drive inflammation, and homeostatic processes that fail to bring inflammation under control. There are increases in the number of both Tregs and Tfh, but in the face of continuing viral replication, the feedback regulation by Tregs does not prevent the florid hyperplasia associated with increased numbers of Tfh and GC B-cells. ART may ameliorate the lymphocyte activation mostly, but not completely. Therapeutic strategies aimed at limiting Tfh activity, or modulating Tregs, should be investigated for potential benefits to boost CD4 reconstitution without unduly boosting Tfh and B-cell hyper-reactivity, or Treg activity.

However, the aim of therapeutic interventions will require very careful consideration due to the complexity of the roles of Tfh and Tregs in pathogenesis. In the case of Tfh, generation of neutralizing antibodies through directed Tfh and B-cell vaccination is a highly desirable outcome (98, 99), but this must be balanced by avoiding excessively increased activation of CD4 T cells and additional GCs as reservoirs of HIV. Similarly, increased Treg activity under HAART may be advantageous in reducing atherosclerosis (220) given the known increased risk of cardiovascular disease in HIV patients, associated with increased inflammation (221), but must be balanced against a need for improved immune reconstitution. Only very detailed studies of these processes will allow rational development of optimal therapy.

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Down regulation of the TCR complex CD3ζ-chain on CD3+ T cells: a potential mechanism for helminth-mediated immune modulation

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The CD3ζ forms part of the T cell receptor (TCR) where it plays an important role in coupling antigen recognition to several intracellular signal-transduction pathways leading to T cell effector functions. Down regulation of CD3ζ leads to impairment of immune responses including reduced cell proliferation and cytokine production. In experimental models, helminth parasites have been shown to modulate immune responses directed against them and unrelated antigens, so called bystander antigens, but there is a lack of studies validating these observations in humans. This study investigated the relationship between expression levels of the TCR CD3ζ chain with lymphocyte cell proliferation during human infection with the helminth parasite, *Schistosoma haematobium*, which causes uro-genital schistosomiasis. Using flow cytometry, peripheral blood mononuclear cells (PBMCs) from individuals naturally exposed to *S. haematobium* in rural Zimbabwe were phenotyped, and expression levels of CD3ζ on T cells were related to intensity of infection. In this population, parasite infection intensity was inversely related to CD3ζ expression levels ($p < 0.05$), consistent with downregulation of CD3ζ expression during helminth infection. Furthermore, PBMC proliferation was positively related to expression levels of CD3ζ ($p < 0.05$) after allowing for confounding variables (host age, sex, and infection level). CD3ζ expression levels had a differing relationship between immune correlates of susceptibility and immunity, measured by antibody responses, indicating a complex relationship between immune activation status and immunity. The relationships between the CD3ζ chain of the TCR and schistosome infection, PBMC proliferation and schistosome-specific antibody responses have not previously been reported, and these results may indicate a mechanism for the impaired T cell proliferative responses observed during human schistosome infection.

Keywords: T cells, CD3ζ, schistosomiasis, human, helminth, downmodulation, antibody, pathology

INTRODUCTION

The T cell receptor (TCR) complex, comprising of the TCR, a CD3ζ chain (CD3ζ) and CD3 co-receptor, has a tightly controlled assembly and expression within cells (1). CD3ζ is an integral part of the signaling pathway involved in TCR signaling (2), and its downregulation has been reported in numerous pathologies and conditions associated with chronic inflammation whilst the TCR on the cell surface remains present at normal concentrations (3). Thus, the CD3ζ chain is considered a "sensor" of sustained exposure to chronic inflammatory immune responses, a mechanism to restrict the magnitude of T cell responses and counteracting an overzealous immune reaction (3). Thus far there have been reports of a downregulated CD3ζ chain in pathologies such as cancers, arthritis, systemic lupus erythematosus, HIV, and leprosy, all conditions associated with impaired T cell functions (3–6).

The helminth parasite *Schistosoma haematobium* causes uro-genital schistosomiasis; a chronic condition typically associated with downregulated immune responses (7). Chronic inflammation associated with schistosomiasis is a hallmark of pathology (8, 9), and experimental models suggest that tissue inflammation is largely CD4+ T helper (Th) 2 driven (10, 11). It is unclear whether the TCR complex is modified in schistosomiasis in the same way as in the Th1 polarized inflammatory diseases in which CD3ζ has previously been studied (3). The immune response to schistosome infection typically consists of elevated levels of regulatory cytokines such as IL-10 and TGFβ (12, 13) and results in

downregulation of T cell proliferation (14), cytokine production (15, 16), and hyporesponsiveness (17). Such a modulated immune response is characteristic of infected individuals in endemic environments who are believed to tolerate infection, facilitating parasite persistence, while at the same time limiting pathology associated with eggs laid by adult worms (18–20). The decreased proliferative capacity of peripheral blood mononuclear cells (PBMCs) during schistosome infection has been reported in both human and experimental studies (21–23), and serves to minimize pathology associated with host inflammatory responses (16). However, this downregulation and suppression of immune responses can also have spill-over effects into other areas of the immune response. For example, helminths are known to affect the host's ability to mount an effective immune response following vaccination, leading to vaccine failure (24, 25). Mechanisms for downregulating the immune response in helminth infection have been investigated in experimental models, and focus primarily on myeloid cells and T regulatory cells (16, 26). Mechanisms associated with control and downregulation of the human immune response have generated interest from the fields of vaccine research, as well as of autoimmunity and allergy due to the potential for therapeutic interventions for these conditions (27–29).

We describe here levels of CD3ζ expression on T cells, PBMC proliferation, and antibody responses from a cohort of individuals living in a schistosome endemic area of rural Zimbabwe. We hypothesized that CD3ζ expression may be downregulated in chronic schistosomiasis and thus be related to *S. haematobium* infection levels within the cohort. Furthermore, we relate CD3ζ expression to schistosome-specific antibodies commonly associated with protection or susceptibility to infection. Our study is the first to show that CD3ζ expression on T cells is reduced during schistosome infection suggesting that this may be a mechanism for immune suppression in schistosomiasis.

MATERIALS AND METHODS

ETHICAL APPROVAL

Ethical and institutional approval was granted by the Medical Research Council of Zimbabwe and the University of Zimbabwe's Institutional Review Board. Local permission for the study was granted by the Provincial Medical Director. The study design, aims, and procedures were explained in the local language, Shona, prior to enrollment. Participants were free to drop out of the study at any time and informed written consent/assent was obtained from all participants and/or their guardians prior to taking part in the study and to receiving antihelminthic treatment.

STUDY DESIGN

The study presented here was part of a larger on-going immuno-epidemiological study based in Mashonaland East, Zimbabwe where *S. haematobium* is endemic as is described elsewhere (30). The area has a low prevalence of soil transmitted helminths (STH) and *Schistosoma mansoni* (31), and the residents are subsistence farmers with frequent contact with *S. haematobium* infected water for purposes of bathing, washing, and collecting water. Recruitment into the study was school based and the wider community was also invited to participate. Residential history, antihelminthic treatment history, and water contact habits of the participants were captured through questionnaire. Following sample collection, participants were offered treatment with the antihelminthic drug praziquantel at the recommended dose of 40 mg/kg of body weight (32).

INCLUSION CRITERIA

In order to be included in this study participants had to meet the following criteria: (1) be lifelong residents of the study area to allow age to be used as a proxy for history of exposure to schistosome infection, (2) have provided a minimum of two urine and two stool samples on consecutive days for parasite detection, (3) not have previously received antihelminthic treatment, (4) be negative for co-infection with malaria, STH, *S. mansoni*, and HIV, and (5) have provided a blood sample for serological and cellular assays. From an initial cohort of 633 recruited individuals, 68 were excluded for not meeting criteria 1–4 above and a further 184 did not provide sufficient blood sample for both serological assays and cell phenotyping. From the remaining 381 individuals, a cohort of 100 individuals was further selected to allow for, as far as possible, equal numbers of females to males and an even distribution of ages and infection prevalence. The final study group was dependent on the participant's PBMC sample yielding at least 10^6 cells to allow enough cells for all experimental conditions. The final study group consisted of 94 individuals and was divided into three age groups as described in **Table 1**.

SAMPLE COLLECTION

From each participant, a stool and urine sample was collected on three consecutive days and examined microscopically for the presence of *S. haematobium* eggs in urine, and *S. mansoni* and STH eggs in stool using standard techniques (33, 34). Up to 20 ml of venous blood was collected from each participant in heparinized tubes and silicone-coated tubes (both from BD Biosciences, San Jose, CA, USA), for collecting PBMCs, and serum. An additional drop of blood was collected from each participant for microscopic detection of malaria parasites and for HIV detection using DoubleCheckGold™ HIV 1&2 Whole Blood Test (Orgenics Ltd., Yavne, Israel). PBMCs were isolated from the remaining tubes via density gradient centrifugation using Lymphoprep™(Axis-Shield, Cambridgeshire, UK). Isolated PBMCs were cryopreserved and stored in liquid nitrogen in Zimbabwe prior to freighting to Edinburgh in dry shippers.

ANTIBODY ASSAYS

Schistosome soluble worm antigen preparation (SWAP) specific antibody serum levels for IgA, IgE, and IgG4 were quantified using antibody ELISA. Lyophilized SWAP (Theodor Bilharz Institute, Giza, Egypt) was reconstituted as described by Mutapi et al. (35). ELISAs were conducted as previously reported (36), adding sample at a 1:20 dilution for IgA and IgE, and 1:100 dilution for IgG4 in 5% (weight/volume) skimmed milk powder. Secondary IgA HRP-conjugated antibody (A-7032 Sigma, St Louis, MO, USA) was added at a 1:1000 dilution, a 1:250 dilution for IgE (P-295, Dako, Glostrup, Denmark), and at a 1:500 dilution for IgG4 (MCA517, AbD Serotec, Oxford, UK). The colorimetric reaction was quantified with an ELISA reader at 405 nm. Each antibody ELISA was performed in duplicate on the same day for all samples with

Table 1 | Characteristics of study cohort.

Infection status	Age group					
	5–10 years		11–15 years		>16 years	
	Sh−	Sh+	Sh−	Sh+	Sh−	Sh+
Sample size (no.)	25	15	17	13	15	9
Mean age in years (range)	7.76 (5–10)	7.60 (5–10)	13.06 (11–15)	12.54 (11–14)	29.73 (16–49)	27.11 (16–54)
Infection intensity	0	60.32	0	99.44	0	78.33
Infection range (SD)	0	1.33–185 (85.40)	0	0.33–523 (165.7)	0	0.33–550 (177.79)
Males:Females	8:17	11:4	9:8	8:5	2:13	3:6

All selected people of the cohort were negative for HIV, soil transmitted helminths and S. mansoni; infection intensity: eggs/10 ml urine; Sh−, negative for S. haematobium, Sh+, positive for S. haematobium.

positive (high responders) and negative (Europeans who have never traveled to helminth endemic areas) controls included on all plates.

DETERMINING CD3ζ EXPRESSION

Cryopreserved PBMCs were thawed as previously described (37), and resuspended at 5×10^6 cells/ml in PBS. Cells were incubated with 10% FCS at 4°C for 10 min prior to staining for 30 min with PerCP-Cy5.5 conjugated anti-CD3 (clone OKT3 from eBiosciences, San Diego, CA, USA). Cells were permeabilized with permeabilization buffer [made up of 0.1% NaAzide and 0.1% saponin in Dulbecco's PBS (Lonza, Verviers, Belgium)], and incubated with FITC conjugated anti-CD3ζ antibody (clone 6B10.2 from BioLegend, San Diego, CA, USA). At least 50,000 live events were acquired on an LSR II flow cytometer (BD Biosciences, San Jose, CA, USA). Analysis was performed using FlowJo software (TreeStar, USA) and mean fluorescence intensity (MFI) was calculated for CD3 and CD3ζ. CD3ζ expression on T cells was determined according to expression levels on CD3 positive cells, and normalized by subtracting the MFI of CD3 in the CD3 negative population.

PROLIFERATION ASSAY

PBMCs were resuspended at 1×10^6 cells per well, and stimulated with mitogens to induce proliferation. Cells were cultured for a total of 54 h at 37°C together with 50 ng/ml phorbol myristate acetate (PMA) and 1 μg/ml phytohemagglutinin (PHA) (Sigma-Aldrich, Dorset, UK), or cultured with X-VIVO medium as a negative control. After 36 h, the supernatant was removed and replaced with fresh X-VIVO medium containing tritiated thymidine (^{3}H-Thymidine) (Amersham Biosciences, GE Healthcare, Little Chalfont, UK) at a final concentration of 0.1 μCurie/well. After a 18-h incubation at 37°C the plates were harvested and proliferation of the cell populations quantified according to ^{3}H-Thymidine cellular incorporation. Uptake of ^{3}H-Thymidine was quantified using a scintillation counter (Wallac-Perkin Elmer, MA, USA). Proliferation was quantified as counts per minute (cpm), and successful proliferation determined as >1000 cpm after media subtraction.

STATISTICAL ANALYSIS

All statistical analyses were conducted using the statistical package SPSS version 19 (IBM Corp., NY, USA). Data were analyzed using parametric linear regression. The data were transformed in order to meet assumptions of parametric tests. CD3ζ was measured as MFI of CD3ζ on CD3 positive cells after subtracting the CD3ζ MFI of the CD3 negative (non T cells) of the same individual. Final measurement of CD3ζ was square-root transformed. Antibody level (after subtraction of the blank control) was square-root transformed. Proliferation data, expressed as cpm were similarly square-root transformed after subtraction of media control values. Infection intensity was log transformed [$\log_{10}(x+1)$]. Categorical variables were sex (male/female), and age group [5–10 years (age group where infection is rising), 11–15 years (age group where infection is peaking), or >16 years (age group where infection is declining)].

Due to the possibility of gender and age dependent exposure patterns in this population (38, 39), it was necessary to allow for variation due to these factors prior to investigating the relationship of interest (40). The relationship between CD3ζ expression and infection intensity was determined via hierarchical linear regression analysis of infection intensity with CD3ζ expression, allowing for variation due to age and sex before testing for the relationship with infection intensity. The relationship between PBMC proliferation and CD3ζ expression for the whole cohort was determined using a linear regression analysis, allowing for variation due to sex, age group, and infection intensity. The relationship between CD3 and CD3ζ, as well as between SWAP IgA and SEA IgA, and IgE:IgG4 against SWAP was assessed for the whole cohort using a partial correlation analysis controlling for age group, infection intensity, and sex. For all statistical tests $p \leq 0.05$ was considered significant.

RESULTS

CD3ζ CHAIN OF THE TCR IS DOWNREGULATED WITH INCREASING LEVELS OF INFECTION

CD3ζ levels were measured on CD3+ T cells within the Zimbabwean cohort as an indication of activation status of the T cells. There was a significant and negative relationship between the intensity of *S. haematobium* infection and CD3ζ expression (**Figure 1**; **Table 2**). In order to confirm that this change was not related to an overall downregulation of the TCR complex (TCR) but due to a specific decrease in the CD3ζ chain, expression of the TCR co-receptor, CD3, was assessed in relation to CD3ζ

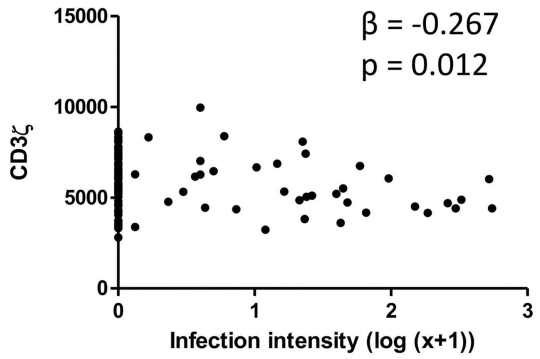

FIGURE 1 | Scatter graph of *Schistosoma haematobium* infection intensity [$\log_{10}$ ($x+1$); *x* axis] and CD3ζ expression on T cells (MFI; *y* axis) for the whole cohort. β and *p* values from linear regression analysis after allowing for age group (5–10, 11–15, 16+ years) and sex (male or female) are shown.

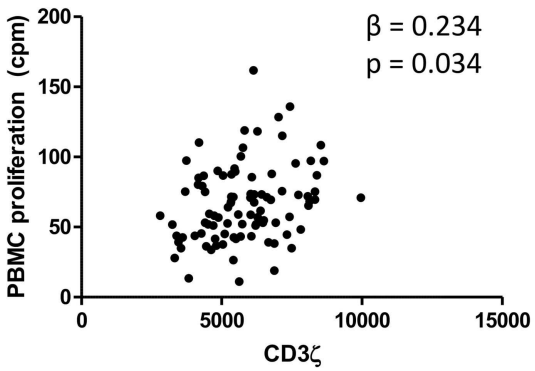

FIGURE 2 | Relationship between CD3ζ expression on T cells (MFI; *x* axis) and PBMC proliferation (cpm; *y* axis) for the whole cohort. β and *p* values from regression analysis after allowing for age group (5–10, 11–15, 16+ years), sex (male or female), and infection intensity [$\log_{10}$ ($x+1$)] are shown.

Table 2 | Coefficients of the regression model relating CD3ζ to schistosome infection intensity.

	Standardized residuals		Unstandardized residuals		95% Confidence interval	
	β Value	*p* Value	Beta	SE	Lower bound	Upper bound
Constant			81.86	3.99	73.93	89.80
Sex	−0.08	0.46	−1.60	2.13	−5.82	2.63
Age group	−0.06	0.59	−0.69	1.27	−0.54	0.59
Infection intensity	**−0.27**	**0.01**	**−3.34**	**1.30**	**−5.93**	**−0.75**

The table shows standardized and unstandardized coefficients from regression analysis. $R^2 = 0.073$. Significant values are shown in bold. β *value for infection intensity is represented on* ***Figure 1****.*
SE, standard error.

expression as well as age and infection status. There was no significant relationship between CD3ζ and CD3 ($r = -0.116$, $p = 0.282$), confirming the relatively independent nature of CD3ζ expression within the TCR (3). Furthermore, the decrease in CD3ζ with infection intensity was not related to total levels of TCR expression, as CD3 levels were not significantly related to infection levels (β = 0.160, $p = 0.124$, see Figure S1 in Supplementary Material).

CD3ζ LEVELS ARE POSITIVELY CORRELATED WITH PBMC PROLIFERATION

Levels of CD3ζ expression on T cells are intrinsically related to TCR activity and downstream immune responses, including cell proliferation (41). We thus related levels of CD3ζ expression on CD3 positive cells to the proliferative capacity of PBMCs from the whole cohort of schistosome exposed individuals. PBMCs from schistosome exposed individuals were stimulated for 54 h with PMA and PHA in order to assess proliferative capacity of the PBMCs independent of schistosome infection. The study showed a significant positive association between PBMC proliferation and CD3ζ expression. The relationship was significant after allowing for variation in sex, age, and infection intensity. **Figure 2** shows the relationship between CD3ζ expression levels and PBMC proliferative capacity after stimulation in this population. CD3 expression was not related to PBMC proliferation (β = −0.025, $p = 0.734$).

Despite CD3ζ expression being significantly related to burden of infection, PBMC proliferation was not significantly related to the burden of schistosome infection (β = −0.168, $p = 0.092$).

CD3ζ LEVELS ARE NEGATIVELY CORRELATED TO PROTECTIVE IMMUNE RESPONSES

Having shown the relationship between CD3ζ and infection intensity, we were interested in whether this was related to immune correlates to infection, as determined by antibody isotypes specific to adult schistosome antigens. Antibody production to the SWAP antigen was measured for IgA, IgG4, and IgE. Previous studies from this population show IgA to be a potential marker for susceptibility to infection (42–44). Relatively high levels of IgG4 are frequently produced in younger ages, thus it is considered to be a marker for susceptibility to infection. In contrast, IgE levels found to be higher in individuals who are more resistant to infection or reinfection (45, 46). Frequently, IgE and IgG4 are compared as ratios to better reflect changes in immunity on an individual level, such that high IgE:IgG4 is considered a marker for resistance to reinfection (45). As shown in **Table 3**, after controlling for sex and age group, IgG4 was found to be positively associated with infection intensity, while IgE and IgA did not show any significant relationship with infection intensity. IgE:IgG4 demonstrated a non-significant negative association with infection intensity.

When relating levels of these antibodies to CD3ζ levels, IgA had a significant negative relationship with CD3ζ. There was also a significant negative correlation between IgE:IgG4 with CD3ζ. **Table 4** shows the *r* and *p* values from the correlation analyses that were performed on the whole cohort.

DISCUSSION

Both human and experimental studies have shown diminished cell proliferation in response to schistosome and bystander antigens

Table 3 | *R* values from partial correlation between SWAP specific IgA, IgE, or IgG4 levels and IgE:IgG4 against infection intensity.

	Infection intensity		
	***R* value**	***p* Value**	**df**
SWAP IgA	−0.013	0.901	86
SWAP IgE	0.117	0.277	86
SWAP IgG4	**0.280**	**0.009**	**83**
SWAP IgE:IgG4	−0.203	0.066	81

R values are from partial correlation, controlling for sex, and age group. Significant relationships are indicated in bold.
df, degrees of freedom.

Table 4 | *R* values from partial correlation between SWAP specific IgA, IgE, or IgG4 levels and IgE:IgG4 against CD3ζ expression.

	CD3ζ		
	***R* value**	***p* Value**	**df**
SWAP IgA	**−0.292**	**0.006**	**85**
SWAP IgE	−0.182	0.092	85
SWAP IgG4	0.109	0.322	82
SWAP IgE:IgG4	**−0.254**	**0.022**	**80**

R values are from partial correlation, controlling for sex, age group, and infection intensity. Significant relationships are indicated in bold.
df, degrees of freedom.

during chronic schistosome infection (15, 22, 23, 47). These diminished responses are related to the presence of parasites (22, 48, 49). In limiting immune cell activation, pathology related to schistosome worm antigens is also limited (16, 50). The aim of this study was to investigate the relationship between expression levels of the TCR CD3ζ chain with lymphocyte cell proliferation during human infection with *S. haematobium* to determine if this is a possible mechanism through which T cell functions may be regulated.

CD3ζ levels are reported to be related to T cell responsiveness and proliferative capacity (2, 3, 41), and infection with schistosomes is associated with reduced proliferative responses (15, 16). Here, we investigated changes in the TCR CD3ζ chain in relation to schistosome infection intensity, and show a previously unreported negative association between CD3ζ levels and schistosome worm burden. In addition, we show a positive relationship between CD3ζ expression and PBMC proliferation, confirming the association between CD3ζ expression levels and PBMC proliferative capacity (3, 26). The observed downregulation of CD3ζ expression in conjunction with increasing infection intensity may be indicating a mechanism for downregulation of T cell proliferation in schistosomiasis. Downregulation of the immune response in schistosome infection is an important mechanism for modulating pathological host immune responses associated with parasite eggs; forming a balance between host immunity and successful parasite establishment (20). Indeed, in experimental models, where the ability to downregulate the immune response has been depleted, an influx of inflammatory cytokines results in increased pathological responses (12, 50).

PBMCs proliferation was not related to infection intensity. Both PHA and PMA directly stimulate or activate cells, bypassing the requirement for surface receptor stimulation (51, 52). The differing relationships between PHA/PMA stimulated PBMC proliferation and CD3ζ with infection intensity suggests that infection related downregulation of CD3ζ does not intrinsically impair cell function, and supports evidence of a reversible suppression of cell function related to the presence of schistosome worms (22).

Protective schistosome acquired immunity has been shown in several studies to be associated with high levels of IgE against adult worm antigens, and moreover a high IgE:IgG4 is associated with protection against reinfection (45, 46). In agreement with this, here we observed a negative association between IgE:IgG4 and infection intensity, as well as a positive and significant relationship between IgG4 and infection intensity, consistent with previous observations (53, 54).

In relating levels of CD3ζ to markers for specific schistosome immune responses, we show that IgA against SWAP was negatively related to CD3ζ levels, as was IgE:IgG4 against SWAP. The IgE:IgG4 is associated with developing immunity (45), indicating a potential relationship between developing immunity in conjunction with lower CD3ζ. IgG4 has been reported to be related to pathology (9, 55), thus the relationship of higher levels of CD3ζ with higher IgG4 levels in relation to IgE (low IgE:IgG4) supports the hypothesis that CD3ζ is downregulated as a protective mechanism against parasite related immune damage and may thus be associated with pathological immune responses. In contrast to IgE:IgG4, previous studies from Zimbabwe have shown IgA to be associated with susceptibility to infection, observing decreases in IgA levels with chemotherapeutic treatment (43), as well as lower levels in uninfected adults (42). Schistosomiasis is a disease, which displays dynamic changes in immune correlates throughout the course of infection (42, 56), with individuals living in endemic areas eventually developing protective immunity to infection (57, 58) and an immune profile that is skewed toward a Th2 dominant profile, while individuals who remain infected display a mixed Th1/Th2 environment (44). It is plausible that the downregulated T cell activity in schistosome infected individuals, observed here through decreased CD3ζ expression, may be contributing to the altered profiles seen in infection, not only preventing a pathological immune response, but also helping create an environment conducive to developing immunity toward infection. Further investigation into the relationship between CD3ζ and immune correlates are required to clarify the function of this marker during developing immunity.

Given that chronic schistosome infection can limit T cell responses to vaccine antigens (24, 27), and in previous studies in this cohort both auto-immune inflammation (59) and allergic reactivity (60) were reduced in people with higher schistosome infection, it is possible that the downregulation of the CD3ζ chain we observe may contribute to these phenomena. Indeed, the precise mechanisms leading to this downregulation of the CD3ζ chain in schistosome infection have important implications for fields such as vaccine development, where a fully functioning immune system is required to achieve optimal vaccine efficacy (61).

While we did not investigate the mechanisms leading to the downregulation of CD3ζ expression in schistosome infection, there may be an association with myeloid cells and myeloid derived suppressor cells (MDSC). For example, downregulation of the CD3ζ chain has previously been found to be related to an increase in activated MDSC in both human and murine studies (62, 63). Specifically in chronic schistosome infection, previous studies in this population, as well as elsewhere, have identified myeloid derived dendritic cells (mDCs) as having an altered expression level (37, 64). Furthermore, in experimental models of helminth infection, alternatively activated macrophages have been implicated as having a regulatory role (65). In humans, neutrophils have also been reported to act as modulators of T cell CD3ζ expression via L-arginine metabolism in inflammation and various pathological conditions (66, 67), and may be an area for further investigation in schistosome infection. Further identification of the mechanisms leading to immune suppression observed in helminth infection (22, 23), and the relationship between downregulation of CD3ζ observed here and clinical indices of helminth-mediated pathology is therefore warranted.

Overall, this study has shown a downregulation of CD3ζ levels in conjunction with increasing schistosome infection. Previous reports of CD3ζ expression being related to T cell activity, and the observation that levels of this marker are related to PBMC proliferation identifies downregulation of CD3ζ as a novel mechanism for immunoregulation during helminth infection in humans. Furthermore, we provide evidence of association between CD3ζ and markers for protective immunity. Mechanistic studies, perhaps including *in vitro* cell culture and blocking antibodies, will elucidate if the association is causal and also if elevated levels of CD3ζ expression mediate, or are a marker of, protective immunity.

AUTHOR CONTRIBUTIONS

Conceived and designed the study: FM, TM, and NM. Contributed to fieldwork: CB, NN, LA, TM, NM, and FM. Designed the experiments: JA, LA, NN, and FM. Performed the experiments: LA and FH. Analyzed the data: LA and FM. Wrote the paper: LA. Contributed to final version of the manuscript: LA, FM, CB, and NN.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/Journal/10.3389/fimmu.2015.00051/abstract

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Human and mouse CD8$^+$CD25$^+$FOXP3$^+$ regulatory T cells at steady state and during interleukin-2 therapy

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In addition to CD4$^+$ regulatory T cells (Tregs), CD8$^+$ suppressor T cells are emerging as an important subset of regulatory T cells. Diverse populations of CD8$^+$ T cells with suppressive activities have been described. Among them, a small population of CD8$^+$CD25$^+$FOXP3$^+$ T cells is found both in mice and humans. In contrast to thymic-derived CD4$^+$CD25$^+$FOXP3$^+$ Tregs, their origin and their role in the pathophysiology of autoimmune diseases (AIDs) are less understood. We report here the number, phenotype, and function of CD8$^+$ Tregs cells in mice and humans, at the steady state and in response to low-dose interleukin-2 (IL-2). CD8$^+$ Tregs represent approximately 0.4 and 0.1% of peripheral blood T cells in healthy humans and mice, respectively. In mice, their frequencies are quite similar in lymph nodes (LNs) and the spleen, but two to threefold higher in Peyer patches and mesenteric LNs. CD8$^+$ Tregs express low levels of CD127. CD8$^+$ Tregs express more activation or proliferation markers such as CTLA-4, ICOS, and Ki-67 than other CD8$^+$ T cells. *In vitro*, they suppress effector T cell proliferation as well as or even better than CD4$^+$ Tregs. Owing to constitutive expression of CD25, CD8$^+$ Tregs are 20- to 40-fold more sensitive to *in vitro* IL-2 stimulation than CD8$^+$ effector T cells, but 2–4 times less than CD4$^+$ Tregs. Nevertheless, low-dose IL-2 dramatically expands and activates CD8$^+$ Tregs even more than CD4$^+$ Tregs, in mice and humans. Further studies are warranted to fully appreciate the clinical relevance of CD8$^+$ Tregs in AIDs and the efficacy of IL-2 treatment.

Keywords: immunological tolerance, immunotherapy, autoimmunity, T cell biology, immune response

INTRODUCTION

T cell development in the thymus comprises the positive selection of functional T cells capable of supporting adaptive immunity and the elimination of highly self-reactive T cells. The latter process is leaky and some autoreactive effector T cells (Teffs) escape into the periphery where they are regulated by peripheral tolerance mechanisms. In healthy individuals, multiple cell subsets with immunoregulatory properties, among CD4$^+$ and CD8$^+$ T cell or B cell populations, control these potentially harmful Teffs (1–3).

The best characterized of such regulatory cell populations are the natural CD4$^+$CD25$^+$FOXP3$^+$ thymic-derived regulatory T cells (CD4$^+$ Tregs). Their major role in the maintenance of immunological self-tolerance and immune homeostasis (4, 5) is illustrated by the rapid development of autoimmune diseases (AIDs) in normal mice upon their depletion (4) and also by the occurrence of severe AID, allergy, and immunopathology in humans with a mutated FOXP3 gene (6). CD4$^+$ Treg biology has dominated research on regulatory cells in AIDs. More recently, other regulatory cell populations have received attention, and notably CD8$^+$ suppressor cells (CD8$^+$ Tsups) for which evidence that they are involved in AIDs is growing (7–9). However, CD8$^+$ Tsups are less well characterized than CD4$^+$ Tregs.

CD8$^+$ Tsups are functionally defined populations of CD8$^+$ T cells endowed with immunosuppressive functions (10–14). Several subpopulations of CD8$^+$ Tsups have been described based on the expression of CD8αα, CD25, CD38, CD45RA, CD45RO, CD56, FOXP3, CXCR3, LAG-3, CD103, CD122, and/or HLA-G, as well as the absence of CD28 and CD127 (2, 7, 8, 15). The different CD8$^+$ Tsup subsets are multiply involved in the pathophysiology of different AIDs (16). Their suppressive activity in AIDs was first demonstrated in CD8-depleted mice, which were more susceptible to a second induction of experimental autoimmune encephalomyelitis (17). CD8-deficient mice were also more susceptible to relapse of autoimmune arthritis after immunization with self-antigens (18). In non-obese diabetic mice, antigen-specific CD8$^+$ Tsups were able to not only prevent but also reverse type 1 diabetes (15). Notably, in patients with systemic lupus erythematosus (19–21), inflammatory bowel disease (22), or multiple sclerosis (23–26) defective functions and/or reduced numbers of CD8$^+$ Tsups have been reported. CD8$^+$ Tsup biology is also less well known than that of CD4$^+$ Tregs, due in part to their small numbers, which render functional studies difficult.

Only some CD8$^+$ Tsups express FOXP3, the master regulator of CD4$^+$ Treg differentiation and function, as well as CD25, and

will be referred to here as $CD8^+$ Tregs. Many groups are now suggesting that FOXP3 expression might represent a good indicator of a bona fide suppressive function (7, 27–30). Notably, it was demonstrated in lupus-prone mice that silencing FOXP3 with siRNA abrogates the ability of $CD8^+$ Tregs to suppress anti-DNA antibodies (27, 29).

As $CD8^+$ Tregs express CD25, the question of their sensitivity to interleukin-2 (IL-2) is relevant. Stimulation with IL-2 is indeed crucial for $CD4^+$ Tregs. Mice deficient in IL-2 or in IL-2 receptor develop systemic AIDs that have been related to impaired development, survival, and function of $CD4^+$ Treg cells (1, 31). However, the lack of IL-2 signaling effects on $CD8^+$ Tregs has not been specifically evaluated in these models. Nevertheless, it is already known that, like $CD4^+$ Tregs, $CD8^+$ Tregs expand in response to IL-2 treatment, as we previously showed in a clinical trial using low-dose IL-2 in hepatitis C virus-induced vasculitis (32) and type 1 diabetes (33).

We sought to characterize $CD8^+CD25^+FOXP3^+$ Tregs in mice and humans, phenotypically and functionally, at steady state and under IL-2 stimulation *in vitro* and *in vivo*. We show here that $CD8^+$ Tregs are highly suppressive and responsive to IL-2. Our results warrant further study of these bona fide $CD8^+$ Tregs in AID pathophysiology and therapy.

MATERIALS AND METHODS

HUMAN BLOOD SAMPLES

Blood samples from healthy volunteers were obtained from the Etablissement français du sang (EFS) at the Pitié-Salpêtrière Hospital in Paris, France. Informed consent was obtained from each volunteer.

Blood samples from type 1 diabetes patients were obtained from the DF-IL2 trial (clinicaltrials.gov identifier NCT01353833). Patients were treated at the Centre d'Investigation Clinique (CIC)-Paris Est of la Pitié-Salpêtrière Hospital in Paris, France. Details about the clinical trial and the patients' clinical characteristics are described in Hartemann et al. (34). Written informed consent was obtained from all participants before enrolment in the study. The study was approved by the institutional review board of the Pitié-Salpêtrière Hospital, and was done in accordance with the Declaration of Helsinki and good clinical practice guidelines.

MICE

BALB/cJRj and C57Bl/6 mice were from Janvier. Transgenic C57Bl/6 FOXP3-GFP mice that express green fluorescent protein (GFP) in $FOXP3^+$ cells were kindly provided by Dr. Malissen of the Centre d'immunologie de Marseille Luminy (France). CD3 KO mice were from CDTA of Orléans (France). Animals were maintained in our animal facility under specific pathogen-free conditions in agreement with current European legislation on animal care, housing, and scientific experimentation. All procedures were approved by the Regional Ethics Committee on Animal Experimentation No. 5 of the Ile-de-France region (Ce5/2012/031).

Preparation of tissue-infiltrating lymphocytes in mice

Spleen, lymph nodes (LNs) (mesenteric LN, MLN; cervical LN, CLN; pancreatic LN, PaLN), and Peyer's patches (PP) were isolated and dissociated in PBS 3% fetal calf serum (FCS). Pancreas was digested with collagenase/DNase solution in RPMI medium, and filtered as described (35). A Ficoll (Sigma-Aldrich) gradient was used to isolate tissue-infiltrating lymphocytes.

Interleukin-2 treatment in mice

Eight-week-old female BALB/c mice received intraperitoneal injections of 50,000 or 100,000 IU of recombinant human IL-2 (Proleukin, Novartis) or PBS daily for 5 days. Twenty-four hours after the last injection, blood was collected and analyzed by flow cytometry.

FLOW CYTOMETRY

Analysis of cell surface and intracellular markers and FOXP3 expression in mice

Fresh total cells from the respective tissues were directly stained with the following monoclonal antibodies (mAbs) at predetermined optimal dilutions for 20 min at 4°C: CD3-PE, CD8-Alexa700, CD4-HorizonV500, CD127-FITC, and CD25-PeCy7 (eBioscience). Intracellular detection of FOXP3 (FOXP3-E450, eBioscience), CTLA-4 (CTLA-4-APC, eBioscience), Ki-67 (Ki-67-FITC, BD Pharmingen), and Bcl-2 (Bcl-2-PE, BD Pharmingen) was performed on fixed and permeabilized cells using appropriate buffer (eBioscience) (incubation 30 min at 4°C). Cells were acquired on an LSR II flow cytometer (Becton Dickinson) and analyzed using FlowJo software (Tree Star, Inc.). Dead cells were excluded by forward/side scatter gating.

Analysis of cell surface markers and FOXP3 expression in humans

Direct *ex vivo* immunostaining was performed on 50 μL of lithium heparinized fresh whole blood from healthy donors using the PerFix-nc kit (Beckman Coulter) according to the manufacturer's instructions. Briefly, 5 μL of fixative reagent was added to the blood for 15 min at room temperature in the dark, before adding antibodies diluted in 300 μL of permeabilizing reagent for 1 h at room temperature in the dark. Staining was performed with $FOXP3^-$ AF647 (clone 259D), CD25-PE, CD127-PE-Cy7, CD8-Chrome Orange, CD4-Pacific Blue, CD3-FITC, CD103-APC, ICOS-FITC, CD122-FITC, and CCR7-PE mAbs, all from Beckman Coulter. CTLA-4-PeCy7 mAb was from Biolegend. Samples were acquired on a Navios cytometer (Beckman Coulter) and analyses were performed using Kaluza software (Beckman Coulter). Matched mouse isotype control antibodies were used.

Instrument settings (gain, compensation, and threshold) were set with machine software (Navios Software; Beckman Coulter) in conjunction with calibration beads (Flow-set beads, Cytocomp kit, and CYTO-TROL Control Cells). Machine reproducibility was verified with standardized beads (Flow-check).

In mice and in humans, $CD4^+$ Tregs were defined as $CD25^+$ $FOXP3^+$ cells among $CD4^+$ T cells, and effector $CD4^+$ T cells as $FOXP3^-$ cells among $CD4^+$ T cells. $CD8^+$ Tregs were defined as $CD25^+FOXP3^+$ cells among $CD8^+$ T cells, and effector $CD8^+$ T cells as $FOXP3^-$ cells among $CD8^+$ T cells.

Absolute numbers of $CD8^+$ Tregs and $CD4^+$ Tregs in peripheral blood

Briefly, PBMC subsets ($CD4^+$ Tregs, $CD8^+$ Tregs) counts (cells/μL) were established from fresh blood samples using

Flowcount fluorescents beads (Beckman Coulter) as internal standard (33).

pSTAT5 staining procedure

The pSTAT5 staining was assessed using PerFix EXPOSE reagents from Beckman Coulter as previously described (36). Briefly, fresh lithium heparinized whole blood was stained using anti-CD4, anti-CD25, anti-FOXP3, and PE-conjugated anti-phosphorylated STAT5 (Beckman Coulter) antibodies. Blood samples were stimulated with increasing hIL-2 (proleukin, Novartis) concentrations at 37°C for 10 min. Cell surface staining was then performed. Whole blood was incubated for 5 min (37°C, incubator). Samples were fixed for 10 min at room temperature in the dark, using 50 μL of fixative reagent (PerFix EXPOSE). Aliquots were permeabilized using 1 mL of permeabilizing reagent (PerFix EXPOSE), and incubated for 5 min at 37°C. Samples were centrifuged at 300 × *g* for 5 min, and the supernatant was completely discarded by aspiration. Then, intracellular staining with a mixture of 100 μL of staining reagent including PE-anti-pSTAT5 and AF647-anti-FOXP3 antibodies was performed for 30 min (room temperature) and cells were washed with 3 mL of washing buffer (PerFix EXPOSE). The supernatant was completely discarded by aspiration and 300 μL of PBS was added.

IN VITRO SUPPRESSION ASSAY

Four C57Bl/6 FOXP3-GFP mice were sacrificed, and spleen and LN were collected and dissociated in PBS 3% FCS. Cells were stained with CD3, CD4, CD8, and CD25 (as described above) and then sorted using a FACS ARIA cell sorter (Becton Dickinson). $CD4^+GFP^-$ ($CD4^+$ Teffs), $CD4^+CD25^+GFP^+$ ($CD4^+$ Tregs), and $CD8^+CD25^+GFP^+$ ($CD8^+$ Tregs) were collected. The purity of cell preparations exceeded 97%. $CD4^+$ Teffs were cultured in RPMI 1640 medium supplemented with 5% FCS, 2 mmol/L L-glutamine, 100 U/mg/mL penicillin/streptomycin at 5×10^4 cells/well, and variable numbers of regulatory cells were added in the presence of 7.5×10^4 total splenocytes from CD3 KO mice and anti-CD3 (final concentration 0.1 μg/mL, BioXell).

3H-thymidine (1 μCi/well) incorporation was evaluated during the final 16 h of the 3-day culture.

STATISTICAL ANALYSES

Statistical significance was evaluated using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, USA, http://www.graphpad.com) and calculated using the paired *t*-test, Mann–Whitney test (comparison of means, unpaired test, non-parametric test, two-tail *p* value), or one-way ANOVA test, with $p < 0.05$ (*) taken as statistical significance (**$p < 0.01$, ***$p < 0.001$, NS, non-significant).

RESULTS

CHARACTERIZATION AND PHENOTYPE OF $CD8^+CD25^+FOXP3^+$ Tregs

We measured the percentages and absolute numbers of $CD8^+$ Tregs and $CD4^+$ Tregs in human blood and in blood and lymphoid organs from C57Bl/6 and BALB/c mice. A representative gating strategy for $CD8^+CD25^+FOXP3^+$ Treg immunophenotyping by flow cytometry in humans (**Figure 1A**) and in BALB/c mice (**Figure 1B**) is shown in **Figure 1**. In mice, $CD4^+$ Tregs and $CD8^+$ Tregs were solely defined by the co-expression of FOXP3 and CD25. In human and to a lesser extent in mouse, $CD4^+$ Tregs and $CD8^+$ Tregs were also characterized by low levels of CD127 compared to Teffs (Figure S1 in Supplementary Material).

In peripheral blood mononuclear cells (PBMCs) from human healthy donors, the percentage of $CD8^+$ Tregs among $CD8^+$ T cells was variable and below 1% (0.38 ± 0.26, mean ± SD, $n = 37$; **Figure 2A**). It is noteworthy that a wide range of values was observed (0.1–1%) with a quite heterogeneous distribution, since few individuals had markedly higher values (**Figure 2A**). In comparison, the percentage of $CD4^+$ Tregs among $CD4^+$ T cells in human blood was higher and less variable (8.3 ± 1.6) (**Figure 2C**).

In mice, the percentages of $CD8^+$ Tregs among $CD8^+$ T cells were 0.07 ± 0.04 and 0.12 ± 0.07% in peripheral blood of C57Bl/6 and BALB/c (**Figure 2A**). Similar or slightly higher values were observed in spleen and CLN in both mouse strains. When MLN and PP were analyzed, two to threefold higher values were observed as compared with blood values, except for PP in BALB/c mice. In comparison, in these different lymphoid tissues, the percentages of $CD4^+$ Tregs among $CD4^+$ T cells were around 50-fold higher in both C57Bl/6 and BALB/c mice (**Figure 2C**).

These 50-fold differences in percentages of $CD4^+$ Tregs and $CD8^+$ Tregs were also observed when looking at absolute numbers (**Figures 2B,D**) in both human and mouse.

We compared the phenotype of mouse $CD8^+$ Tregs with that of other $CD8^+$ T cells and of $CD4^+$ T cells, focusing mainly on proteins associated with the regulatory function of $CD4^+$ Tregs (**Figure 3**). Most $CD8^+$ Tregs expressed CD103 (85.2 ± 11.8%) and some were $CD122^+$ (11.9 ± 9.6%) with significantly higher levels compared with $CD8^+$ Teffs only for CD103 expression ($p < 0.0001$). Approximately 36% of them were $ICOS^+$ and only 9% expressed more CTLA-4 than $CD8^+$ Teffs ($p < 0.0001$ and $p = 0.0025$, respectively). $CD8^+$ Tregs had a proliferation rate measured by expression of Ki-67 (19.8 ± 11.7%) that was more than three times higher than that of $CD8^+$ Teffs (6.6 ± 2.0%, $p < 0.0001$), whereas anti-apoptotic Bcl-2 marker expression was the same in $CD8^+$ Tregs and $CD8^+$ Teffs. This phenotype was similar in $CD4^+$ Tregs, except for a lower expression of CD103, CD122, and ICOS (**Figure 3**).

A large fraction of human $CD8^+$ Tregs express CTLA-4 (59 ± 19%) (Figure S2 in Supplementary Material) as do $CD4^+$ Tregs. In contrast, $CD8^+$ Tregs do not express any of the other markers we tested, i.e., CD103, CD122, ICOS, and CCR7 (data not shown).

SUPPRESSIVE ACTIVITY OF $CD4^+$ Tregs AND $CD8^+$ Tregs

We next evaluated the suppressive capacity of $CD8^+$ Tregs. For that purpose, we used C57Bl/6 FOXP3-GFP mice. In these knock-in mice, GFP is expressed only in *FOXP3* positive cells and can thus be used as a marker for flow cytometry sorting of viable $CD8^+$ Tregs. $CD8^+$ Tregs ($CD8^+CD25^+GFP^+$) and $CD4^+$ Tregs ($CD4^+CD25^+GFP^+$) were sorted and evaluated for their capacity to suppress $CD4^+$ Teffs ($CD4^+GFP^-$ cells). $CD8^+$ Tregs appeared slightly more suppressive than $CD4^+$ Tregs (**Figure 4**). At a 1:1 Treg:Teff ratio, 95% of $CD4^+$ Teff suppression was observed with $CD8^+$ Tregs and 77% with $CD4^+$ Tregs. Similar differences were observed at a 1:2 ratio in the same experiment and similar results were also obtained with human $CD8^+$ Tregs (28).

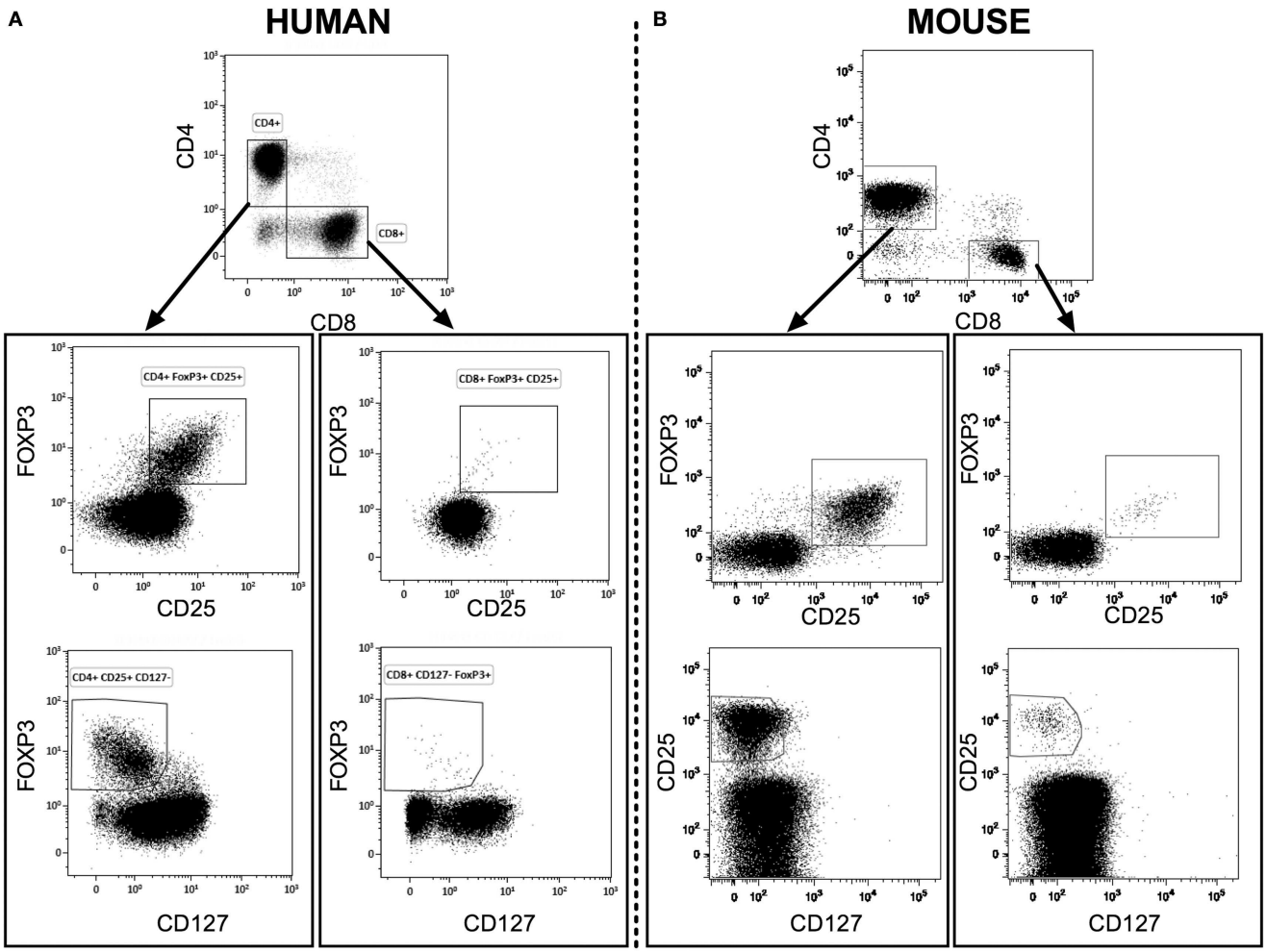

FIGURE 1 | Representative flow cytometry analysis of CD4+ Tregs and CD8+ Tregs from fresh heparinized peripheral blood of human healthy donors [human (A)] and of naive BALB/c mice [mouse (B)]. CD4+ and CD8+ T cells were gated from CD3+ lymphocytes.

IN VITRO SENSITIVITY TO IL-2 OF CD4+ Tregs AND CD8+ Tregs

As $CD4^+$ Tregs and $CD8^+$ Tregs constitutively express CD25, included in the high-affinity IL-2 receptor, we investigated their sensitivity to *in vitro* IL-2 activation. We evaluated the IL-2-induced phosphorylation of STAT5 proteins (pSTAT5) by flow cytometry (**Figure 5**). $CD4^+$ Tregs are exquisitely sensitive to IL-2 activation, activated with doses 20- to 40-fold lower than those required to activate $CD4^+$ Teffs. It is noteworthy that $CD8^+$ Tregs had the same 20- to 40-fold higher sensitivity to IL-2 activation as $CD8^+$ Teffs, but were 2- to 4-fold less sensitive than $CD4^+$ Tregs (**Figure 5A**). At 1 IU/mL, the proportion of pSTAT5 positive cells was of $77.7 \pm 3.8\%$ in $CD4^+$ Tregs, $29.1 \pm 5.2\%$ in $CD8^+$ Tregs, but only 2.9 ± 0.5 and $1.2 \pm 0.3\%$ in $CD4^+$ and $CD8^+$ Teffs, respectively. At 10 IU/mL, these proportions were $91.9 \pm 1.5\%$ for $CD4^+$ Tregs, $46.0 \pm 6.0\%$ for $CD8^+$ Tregs, and only $13.4 \pm 2.4\%$ for $CD4^+$ Teffs and $4.7 \pm 1.7\%$ for $CD8^+$ Teffs. These differences in IL-2-induced activation in $CD4^+$ Tregs and $CD8^+$ Tregs can be correlated with twofold higher levels of CD25 basal expression in $CD4^+$ Tregs compared with $CD8^+$ Tregs (**Figure 5B**).

IN VIVO SENSITIVITY TO IL-2 OF CD4+ Tregs AND CD8+ Tregs

We next assessed the dynamics of $CD4^+$ Tregs and $CD8^+$ Tregs under low-dose IL-2 treatment, which is known to expand and activate $CD4^+$ Tregs. Seven patients with type 1 diabetes received 3 MIU of IL-2/day for 5 days (34). This led to a significant increase of $CD8^+$ Tregs, which reached 6.7 ± 4.3-fold at day 5 (**Figure 6A**). This $CD8^+$ Treg expansion was not sustained at day 15, i.e., 10 days after the last IL-2 injection. IL-2 had a less pronounced effect on $CD4^+$ Tregs, reaching a 1.8 ± 0.7-fold expansion at day 5. Nevertheless, and unlike $CD8^+$ Tregs, this increase was sustained until day 15 (1.8 ± 0.6-fold). Interestingly, CD25 mean fluorescence intensity (MFI) was increased both on $CD8^+$ Tregs and $CD4^+$ Tregs after 5-day IL-2 treatment (**Figure 6B**). This CD25 increase was slightly higher for $CD4^+$ Tregs than for $CD8^+$ Tregs (2.1 ± 0.3 versus 1.6 ± 0.4; $p = 0.005$).

In BALB/c mice injected daily for 5 days with 50,000 or 100,000 IU of IL-2, we observed a similar dose-dependent increase of percentages of $CD4^+$ and $CD8^+$ Tregs in peripheral blood as compared with controls injected with PBS (**Figure 7A**). A

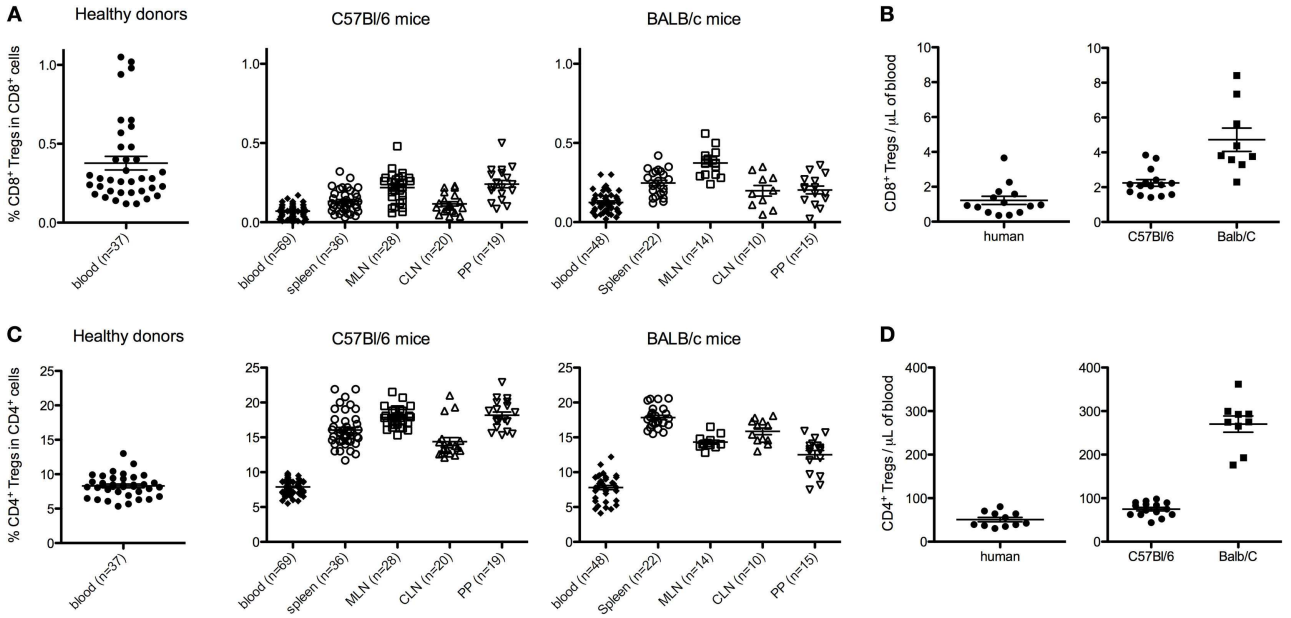

FIGURE 2 | Percentages and absolute counts of CD8+ Tregs among CD8+ T cells [respectively (A,B)], and of CD4+ Tregs among CD4+ T cells [respectively (C,D)] in human healthy donors and C57Bl/6 or BALB/c naive mice. Human data are from 10 to 37 healthy blood donors. Mouse data are from a pool of at least three different experiments analyzing 8- to 12-week-old female mice. Individual values and mean ± SEM are shown. MLN, mesenteric lymph nodes; CLN, cervical lymph nodes; PP, Peyer's patches.

5-day course of 50,000 IU of IL-2 led to a 1.5 ± 0.3-fold increase of $CD4^+$ Tregs and 1.5 ± 0.6-fold increase of $CD8^+$ Tregs. At 100,000 IU/day, the increase was 1.9 ± 0.1-fold for $CD4^+$ Tregs and 1.8 ± 0.5-fold for $CD8^+$ Tregs. Similarly to the human study, we observed an IL-2 dose-dependent increase of CD25 expression in both $CD4^+$ Tregs and $CD8^+$ Tregs (**Figure 7A**). The fold increase of CD25 MFI induced by 50,000 or 100,000 IU of IL-2 was 1.4 ± 0.2 or 1.8 ± 0.2 in $CD4^+$ Tregs and 1.3 ± 0.4 or 1.8 ± 0.5 in $CD8^+$ Tregs, respectively. In contrast, FOXP3 expression levels were slightly increased only in $CD4^+$ Tregs (1.1 ± 0.1 both with 50,000 and 100,000 IU of IL-2). No significant differences in FOXP3 expression level were observed in $CD8^+$ Tregs at the two doses.

We also monitored some activation and proliferation markers in $CD4^+$ Tregs and $CD8^+$ Tregs after a 5-day course of 50,000 IU of IL-2. Interestingly, CD103, CTLA-4, ICOS, Ki-67, and Bcl-2 expression levels were increased after IL-2 treatment in $CD4^+$ Tregs but not in $CD8^+$ Tregs (**Figure 7B** and representative stainings in Figure S2 in Supplementary Material). These results highlight the specific biology of $CD8^+$ Tregs compared with conventional $CD4^+$ Tregs.

DISCUSSION AND CONCLUSION

ON BONA FIDE CD8+ Tregs

$CD8^+$ Tsups are heterogeneous and often poorly characterized if not controversial cells in mice and humans. We focused our study on $CD8^+CD25^+FOXP3^+$ T cells, which we call $CD8^+$ Tregs, because they are identified based on the same markers as $CD4^+$ Tregs. $CD8^+$ Tregs are present at steady state at low levels both in human and mouse peripheral blood (about 0.4 and 0.1%, respectively). We previously reported lower percentages of $CD8^+$ Tregs in blood of normal volunteers (0.22 ± 0.1%) (28), but we were using a different flow cytometry staining strategy. Because we optimized our flow cytometry analyses (choice of antibodies, analyses of whole blood with Perfix-NC kit), we believe that our new values accurately reflect their percentages. Natural $CD8^+CD25^+FOXP3^+$ T cells are also present at very low frequencies (between 0.3 and 1.8% in $CD8^+$ T cells) in blood, LNs, thymus, and spleen from naive non-human primates (37, 38).

We report here that $CD8^+$ Tregs, as $CD4^+$ Tregs, constitutively express CD25 and are sensitive to *in vitro* IL-2 activation, to which they respond by STAT5 phosphorylation. Like $CD4^+$ Tregs, $CD8^+$ Tregs also express markers associated with $CD4^+$ Treg suppressive activity such as ICOS (39), CD103 (40, 41), and CTLA-4 (42, 43) at higher levels than other $CD8^+$ T cells. It is of note that nearly 80% of $CD8^+$ Tregs are $CD103^+$, a marker associated with suppressive function of $CD8^+$ T cells (21, 40, 44). This phenotype is associated with a high suppressive activity *in vitro* in classic functional assays. Similar suppressive activities of $CD8^+$ Tregs have also been reported in humans (28) and non-human primates (45). Altogether, $CD8^+FOXP3^+CD25^+$ T cells share phenotypic and functional characteristics with thymic $CD4^+$ Tregs and are thus bona fide $CD8^+$ Tregs, a designation that should be restricted to this population.

As for $CD4^+$ Tregs, it seems that there could be a population of induced $CD8^+$ Tregs. $CD8^+Foxp3^+$ can be induced *ex vivo* from $CD8^+Foxp3^-$ cells after culture in the presence of IL-2 and TGF-beta, but are only mildly suppressive (45, 46). Following simian immunodeficiency virus (SIV) infection, these peripheral $CD8^+CD25^+Foxp3^+$ regulatory T cells are largely induced

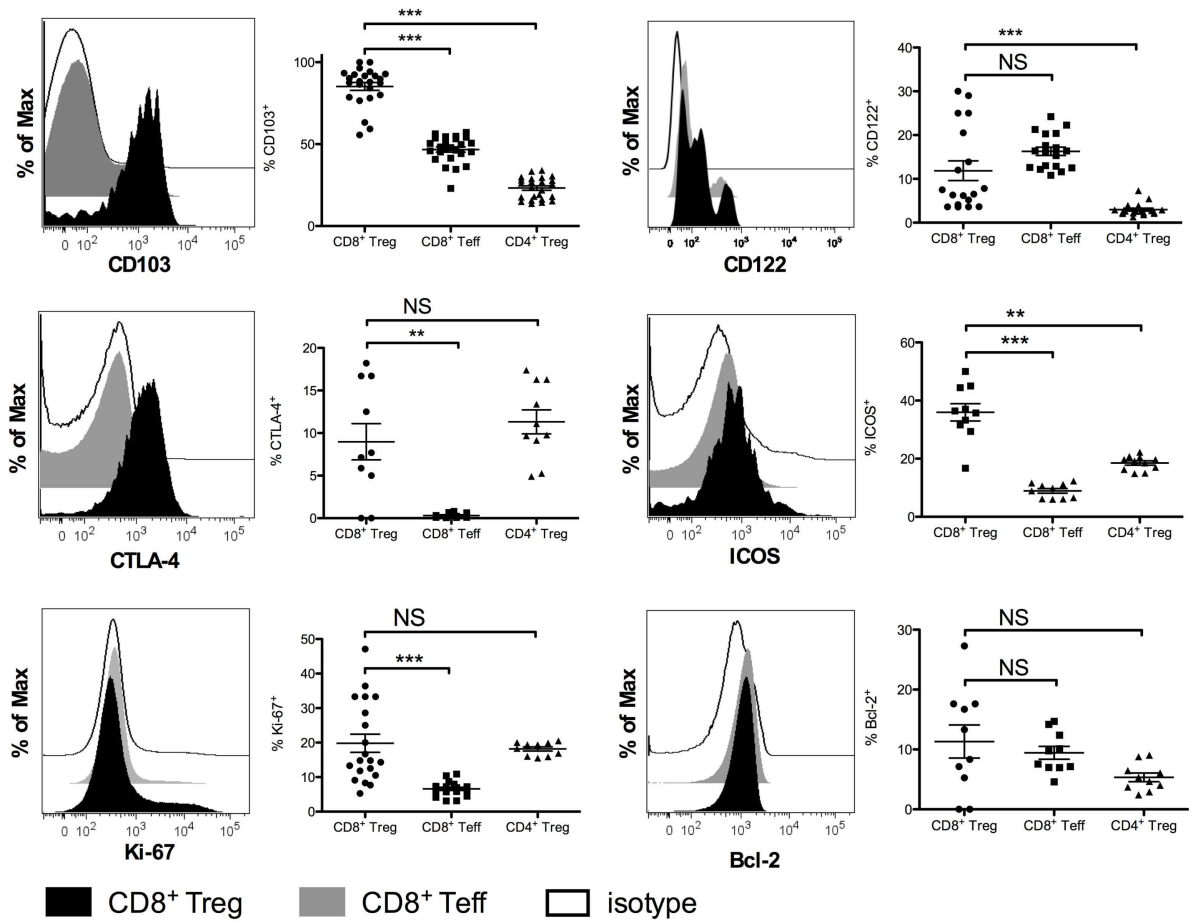

FIGURE 3 | Phenotypic characterization of CD8+CD25+FOXP3+ Tregs (CD8+ Tregs) compared with CD8+CD25−FOXP3− effector CD8+ T cells (CD8+ Teffs) and with CD4+CD25+FOXP3+ Tregs (CD4+ Tregs) in female BALB/c mice. Representative histograms showing expression of CD103, CTLA-4, Ki-67, CD122, ICOS, and Bcl-2 in CD8+ Teffs (gray) and in CD8+ Tregs (black) compared to isotype control (white) are shown. Corresponding percentages in the overall population are shown. Individual values and mean ± SEM are shown.

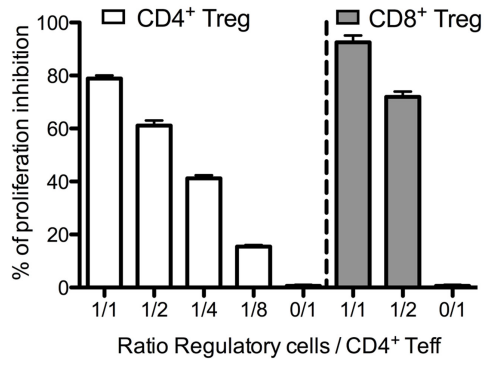

FIGURE 4 | Suppressive activity of murine CD4+ Tregs and CD8+ Tregs. Freshly sorted CD4+GFP− (CD4+ Teffs) cells were cultured with CD4+ Tregs or CD8+ Tregs at various ratios for 3 days, and tritiated thymidine was added for the last 16 h of the culture. Proliferation was assessed by tritiated thymidine incorporation measurement.

not only in blood but also in colorectal mucosa (37, 38). These cells have a regulatory phenotype (CD25+, CTLA-4+, CD28+, CD127−) (26, 28) with high proliferative capacities as 60% of induced CD8+ Tregs are Ki-67+. They express low levels of granzyme B and perforin, suggesting that their suppression is not mediated by killing.

ON DIFFERENCES BETWEEN CD4+ Tregs AND CD8+ Tregs

While CD8+ Tregs and CD4+ Tregs share functional suppressive activity, differences in their biology have been reported. IL-2 can bind the high-affinity trimeric IL-2 receptor (IL-2R) (CD25, CD122, and CD132) or the low-affinity dimeric IL-2R (CD122 and CD132) (47, 48). The trimeric IL-2R is constitutively expressed on CD4+ Tregs, but only transiently expressed on CD4+ and CD8+ T cells following T cell receptor (TCR) activation (47). We show here that CD8+ Tregs constitutively express the trimeric IL-2R, but compared with CD4+ Tregs, CD8+ Tregs express fewer CD25 molecules on their cell surface. CD4+ Tregs are two to fourfold more sensitive to IL-2 activation than CD8+ Tregs, although both CD4+

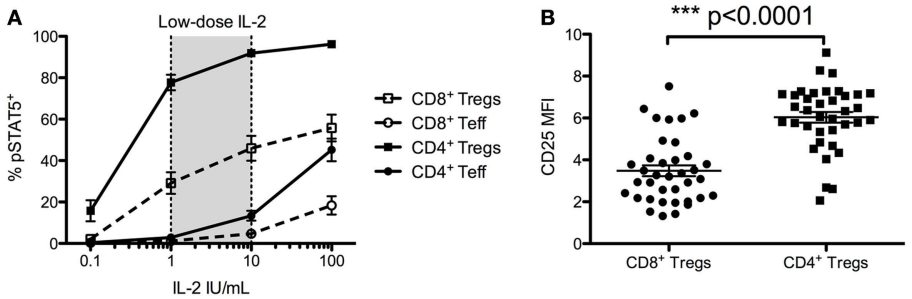

FIGURE 5 | Foxp3$^+$ CD25^{high} CD127^{low} CD4$^+$ T cells (CD4$^+$ Tregs) and Foxp3$^+$ CD25^{high} CD127^{low} CD8$^+$ T cells (CD8$^+$ Tregs) responsiveness to IL-2 and their comparative CD25 cell surface expressions. **(A)** Total fresh blood from healthy donors was *ex vivo* stimulated with different doses of IL-2 for 10 min, and STAT5 phosphorylation was measured on different T cell subsets. Results are expressed in mean ± SE, $n = 7$–22. Low-dose IL-2 has been defined as the dose inducing high STAT5 phosporylation on CD4$^+$ Tregs but not on CD4$^+$ Teff. **(B)** Mean fluorescence intensity (MFI) of CD25 on CD4$^+$ Tregs and CD8$^+$ Tregs ($n = 37$) from fresh unstimulated whole blood from healthy donors. Individual values and mean ± SEM are shown.

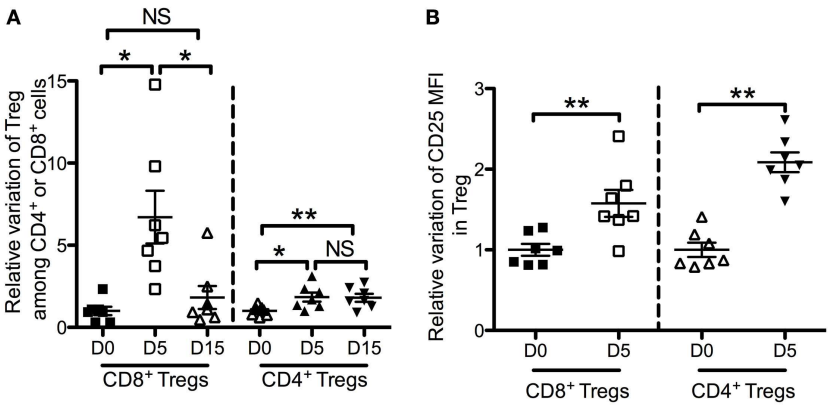

FIGURE 6 | Effects of low-dose IL-2 on Foxp3$^+$ CD25^{high} CD127^{low} CD4$^+$ T cells (CD4$^+$ Tregs) and Foxp3$^+$ CD25^{high} CD127^{low} CD8$^+$ T cells (CD8$^+$ Tregs) in humans. Seven type 1 diabetes patients were treated with 3 MIU IL-2/day for 5 days (34). Time-course changes of CD8$^+$ Tregs and CD4$^+$ Tregs percentages in peripheral blood **(A)** and of CD25 mean fluorescence intensity (MFI) in CD8$^+$ Tregs and CD4$^+$ Tregs in peripheral blood **(B)** following a 5-days course of low-dose IL-2. Individual values and mean ± SEM are shown. Results are expressed in fold variation compared with the mean at day 0 of all the samples.

and CD8$^+$ Tregs are equally 20 times more sensitive to IL-2 than their FOXP3$^-$ counterparts. However, despite these differences in favor of CD4$^+$ Tregs *in vitro*, we observed that the sensitivity of CD8$^+$ Tregs to IL-2 *in vivo* is higher than that of CD4$^+$ Tregs (**Figure 6A**), a so far unexplained observation. In agreement with our observations, a very large increase of CD8$^+$ Tregs, greater than that for CD4$^+$ Tregs, has been observed in blood of non-human primates following IL-2 treatment (49) in a context of tuberculosis infection.

Upon IL-2 activation, CD25 up-regulation is lower for CD8$^+$ than for CD4$^+$ Tregs. Also, after IL-2 treatment, activation markers such as CD103, CTLA-4, and ICOS, which are increased in CD4$^+$ Tregs, are not increased in CD8$^+$ Tregs, suggesting that IL-2 regulates CD8$^+$ Tregs differently than CD4$^+$ Tregs. Further studies are needed to clarify the molecular effects of IL-2 on CD8$^+$ Tregs.

Importantly, we showed that CD8$^+$ Tregs suppress *in vitro* effector T cell proliferation as well as or even better than CD4$^+$ Tregs. This experiment has been performed only in mice as for now the low number of bona fide CD8$^+$ Tregs in peripheral blood from human healthy donors does not allow to perform suppressive assays with these cells. However, we have already showed that non-manipulated human cord blood CD8$^+$ Tregs are suppressive (50). It has also been reported that CD8$^+$ Tregs from healthy non-human primates suppress as well as CD4$^+$ Tregs (45). Thus, we can assume that, even if CD8$^+$ Tregs are less numerous than CD4$^+$ Tregs in peripheral blood of human healthy donors, their excellent suppressive activity makes them important cells to consider in the regulation of immune responses.

For now, it has previously shown by others and us that *in vivo* IL-2 expanded CD4$^+$ Tregs have better *in vitro* suppressive activities than non-expanded CD4$^+$ Tregs. It remains to evaluate whether this is also true for CD8$^+$ Tregs functions.

ON THE POSSIBLE ROLE OF CD8$^+$ Tregs

Several mechanisms have been implicated in the suppressive activity of suppressive CD8$^+$ T cells, including direct lysis of target cells and secretion of immunosuppressive cytokines (14).

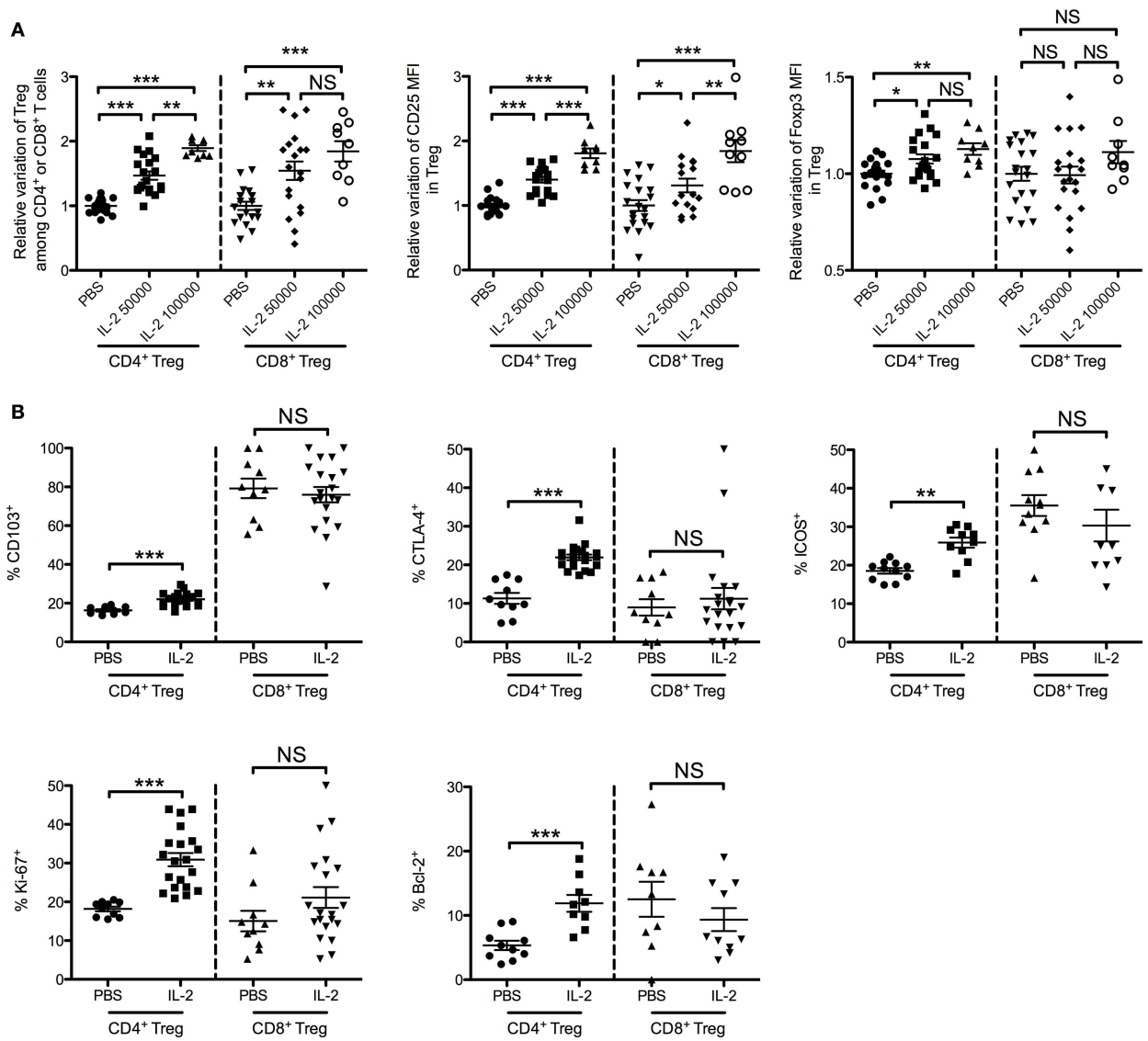

FIGURE 7 | Effects of low-dose IL-2 on CD4+ Tregs and CD8+ Tregs in mice. Eight-week-old female BALB/c mice were injected daily for 5 days with PBS or with 50,000 or 100,000 IU of IL-2, and blood was sampled after the treatment. **(A)** Relative variations of percentages, CD25 MFI and FOXP3 MFI of CD4+ Tregs and CD8+ Tregs in blood after treatment (pool of three independent experiments). Individual values and mean ± SEM are shown. **(B)** Phenotypic activation markers of CD4+ Tregs and CD8+ Tregs after 5-day course of PBS (PBS) or 50,000 IU of IL-2 (IL-2) (pool of two independent experiments). Individual values and mean ± SEM are shown.

CTLA-4 has been shown to play a role in cell–cell contact-dependent mechanisms of $CD4^+$ Tregs and $CD8^+$ Treg-mediated suppression (51). As $CD4^+$ Tregs, $CD8^+$ Tregs may impede the up-regulation of CD80 and CD86 on immature dendritic cells or down-modulate them on mature dendritic cells (42) via CTLA-4, thereby inhibiting activation of Teffs. Jebbawi et al. (50) described a similar natural $CD8^+$ Treg population from cord blood. This population expresses CTLA-4 and can secrete IL-10 and TGF-beta compared with $CD8^+CD25^-$ T cells.

$CD8^+$ Tregs have also been implicated in the regulation of human AID, including inflammatory bowel disease (22). Eusebio et al. (52) showed also that asthma patients have fewer $CD8^+$ Tregs in blood than healthy control subjects. Furthermore, FOXP3 mRNA levels of $CD8^+$ T cells were significantly decreased in patients with severe asthma compared with mild to moderate asthma and control patients. Thus, natural $CD8^+$ Tregs could play a major role in control of allergic inflammation.

In cancer, Chaput et al. (28) and Kiniwa et al. (53) demonstrated that $CD8^+$ Tregs from colorectal and prostate tumors have strong immunosuppressive properties, and may contribute to tumoral immune escape and disease progression.

As many groups have reported the presence of $CD8^+$ Tregs after induction either by a drug or by pathological conditions (AIDs, cancer, infection, allergy), it remains to be seen in these models

whether they are induced by pathological or natural conditions. In any case, we demonstrate here that bona fide $CD8^+$ Tregs are present at steady state in mice and humans.

CONCLUSION

Many questions remain, especially about the origin and role of natural $CD8^+$ Tregs in health and disease. Their striking phenotypic similarities with $CD4^+$ Tregs, robust suppressive activity *in vitro* and exquisite sensitivity to IL-2 warrant further studies to fully appreciate their clinical relevance in AIDs and inflammatory diseases, and in IL-2 treatment efficacy.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fimmu.2015.00171

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