Regulatory T Cells As Potential Targets for HIV Cure Research

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T regulatory cells (Tregs) are a key component of the immune system, which maintain a delicate balance between overactive responses and immunosuppression. As such, Treg deficiencies are linked to autoimmune disorders and alter the immune control of pathogens. In HIV infection, Tregs play major roles, both beneficial and detrimental. They regulate the immune system such that inflammation and spread of virus through activated T cells is suppressed. However, suppression of immune activation also limits viral clearance and promotes reservoir formation. Tregs can be directly targeted by HIV, thereby harboring a fraction of the viral reservoir. The vital role of Tregs in the pathogenesis and control of HIV makes them a subject of interest for manipulation in the search of an HIV cure. Here, we discuss the origin and generation, homeostasis, and functions of Tregs, particularly their roles and effects in HIV infection. We also present various Treg manipulation strategies, including Treg depletion techniques and interventions that alter Treg function, which may be used in different cure strategies, to simultaneously boost HIV-specific immune responses and induce reactivation of the latent virus.

Keywords: regulatory T cells, FoxP3, human immunodeficiency virus, simian immunodeficiency virus, lymph node, virus eradication, cytotoxic T lymphocytes

INTRODUCTION

The human immune system walks a fine line between protection from pathogens and self-reactivity. These functions are mediated by both the innate and adaptive immune responses, such that all immune cells, from monocytes and natural killer cells to B and T lymphocytes, play integral roles in protection. Yet, a major function of the immune system, regulation, and self-tolerance, was not well understood for a long time. Gershon and Kondo in 1970 first described a population of thymus-derived lymphocytes, which were responsible for the induction of tolerance in bone marrow-derived lymphocytes (1). However, the mechanism of action and the cellular characteristics of these cells were not studied in detail until 1995, when Sakaguchi et al. reported that the CD25hi CD4+ T cell subset has an immunoregulatory function and helps defend against the development of autoimmunity, rekindling the interest in this regulatory population (2). These cells have since been termed T regulatory cells (Tregs), and their immunosuppressive functions have been extensively investigated over the past 23 years. We now understand that Tregs are essential...
for proper homeostasis of the immune system and regulation of self-tolerance. While clearly playing a significant role in the pathogenesis of HIV infection, there is still debate in the field of whether Tregs are a boon or bane to fighting the virus. More recently, Tregs were reported to be involved in the HIV reservoir seeding, maintenance, and control of reactivation. In this review, we discuss Tregs and their roles during HIV infection, with emphasis on their role in viral persistence, and strategies for Treg manipulation that may have an impact for an HIV cure.

**TYPES OF Tregs**

Based on their site of differentiation, Tregs can be classified into thymic Tregs (tTregs) and peripheral Tregs (pTregs). The differences in the differentiation of tTregs and pTregs have been described in detail by Lee et al (3).

Separation based on their immunophenotypes identified numerous distinct Treg subpopulations (4) (Table 1). Treg classification through other methods, such as mass cytometry, also showed that they form a very diverse population, with up to 22 different Treg subsets being identified (5). In this section, we only focus on the key Treg subsets for which both immunophenotypes and function were well characterized.

Forkhead box P3 (FoxP3) is the key marker and master regulator of Tregs (6). In fact, Tregs are defined to have a CD25hi FoxP3+ CD4+ phenotype. The importance of this protein was discovered when mutations in the foxp3 gene that codes for FoxP3 were shown to cause the X-linked recessive disease, scurfy, in mice. Scurfy presents as lymphoproliferation leading to fatal autoimmunity, and mimics X-linked autoimmunity-allergic dysregulation syndrome in humans (7). Scurfy mice administered with stable Tregs, defined by FoxP3 expression and full suppressive functionality, did not develop any signs of the disease (8). FoxP3 expression can also be transiently induced following in vitro stimulation of nonsuppressive CD25neg CD4+ T cells, which indicates that expression of FoxP3 alone is not responsible for the regulatory activity of T cells (52).

Thymic Tregs are defined by the expression of CD25 and FoxP3 on CD4+ T cells. It has been shown that CD25hi CD4+ Treg cells develop from self-reactive thymic cells that express a T cell receptor (TCR) with high affinity for self-antigens. Differentiation occurs as an alternative mechanism to apoptosis, such that self-antigen reactivity can induce an inhibitory response instead of an autoimmune response (53). Upon TCR interaction with these peptide-major histocompatibility complex (MHC) complexes, FoxP3 is induced in the immature thymocytes (54). However, FoxP3 expression is not sufficient to create a stable Treg. Demethylation of the FoxP3 locus in the Treg-specific demethylated region (TSDR) is required to generate stable tTregs (55). In addition, CpG hypomethylation of certain loci called ”Treg cell representative regions” is imprinted in Tregs, also contributing to their stability (56). Interactions between B7 molecules (CD80 and CD86), expressed on the antigen-presenting cells (APCs), and CD28, on T lymphocytes, are co-stimulatory and are critical to the thymic development of Tregs, as evidenced by the severe decrease in Treg numbers in mice either deficient in CD28 or treated with a blocking anti-B7 antibody (15, 57, 58). Interleukin-2 (IL-2), the central cytokine involved in Treg biology, is also essential for tTreg maturation (59).

**TABLE 1 | Key immunophenotypic markers/molecules and cytokines expressed by T regulatory cells (Tregs) and their function.**

| Marker/molecule | Function in Tregs | Reference |
|----------------|-------------------|-----------|
| CD25 | Receptor for IL-2, essential for Treg function and maintenance | (2) |
| FoxP3 (forkhead box P3) | Co-ordinates expression of various genes required for Treg activity | (6–8) |
| CD127 (Low) | Receptor for IL-7 | (9, 10) |
| CTLA-4 (Cytotoxic T lymphocyte antigen-4)/CD152 | Ablates CD28 costimulatory binding to CD80 and CD86. | (11–14) |
| CD28 | Upregulation of IDO production by DCs | |
| PDL1 (Programmed cell death-1) | Development and maturation, activation induced Treg markers and expression of CCR6 | (15, 16) |
| ICOS (Inducible costimulator)/CD278 | Binds to PD-L1, inhibits proliferation and effector responses of lymphocytes | (17–19) |
| LAG-3 (Lymphocyte activation gene-3)/CD223 | Controls expansion and maintenance of the Foxp3+ regulatory T cells, and IL-10 production | (20–22) |
| GITR (Glucocorticoid-induced tumor necrosis factor receptor)/CD157 | Plays an important role during IL-27-mediated enhanced Treg function | (23) |
| GARP (Glycoprotein A repetitions predominant) | Differentiation of thymic Tregs (tTregs), and expansion of both Tregs and pTregs | (24, 25) |
| TNRF2 (tumor necrosis factor receptor 2)/CD120b Helios | Present on activated Tregs; promote activation and secretion of TGF-β | (26, 27) |
| | Promotes sustained expression of FoxP3 | (28) |
| | Highly expressed on Tregs; enhances Treg function by increasing expression of other Treg functional molecules | (29–32) |
| CD39 | Anti-inflammatory effect by hydrolytically cleaving ATP to AMP | (33, 34) |
| CD73 | Anti-inflammatory effect by hydrolyzing AMP cleaved by CD39 to adenosine | (35, 36) |
| CCR4 | Expressed on effector Tregs; required for recruitment to tissue, through CCL22 | (37–38) |
| CCR6 | Regulates migration to inflammatory tissue | (39) |
| CCR7 | Required for migration to lymph nodes; limits Treg circulation back to the thymus | (40–42) |
| CXCR5 | Expressed on Tfr cells; required for homing to the germinai centers | (43) |
| Cytokines | | |
| IL-10 | Secreted; anti-inflammatory | (44, 45) |
| TGF-β | Membrane bound and secreted; suppressive; important for Treg trafficking to the gut | (46–50) |
| IL-35 | Secreted; suppressive | (51) |
In addition to Tregs, it has become clear that de novo expression of FoxP3 can occur in non-Treg CD4+ T cells, either in vitro or in vivo. Such induction of FoxP3 expression notably happens when naïve T cells are stimulated in the presence of transforming growth factor beta-1 (TGF-β1) (60), leading to the development of a subset of induced Tregs (iTregs). This subset has extensively been used to study the functions and characteristics of Tregs ex vivo (61); however, it is now recognized that these in vitro-induced iTregs may not accurately portray the characteristics of in vivo-induced, pTregs. Notably, full FoxP3 TSDR methylation does not occur in TGF-β-induced Tregs, leading to poorly suppressive and unstable Tregs (62). Conflicting reports have been published with regard to the contribution of retinoic acid (RA) to pTreg differentiation. RA from dendritic cells (DCs) was reported to be a key cofactor in generating pTregs in the gut (63, 64). However, supplementation by RA does not increase Treg frequency (65), which has cast doubt on the role really played by RA in pTreg differentiation. Interestingly, RA can phosphorylate AKT (protein kinase B) (66), thereby reducing its activity, and this pathway could be involved in pTreg differentiation, because a constitutively active AKT has been shown to impair de novo induction of FoxP3+ cells (67). Another pathway involved in pTreg induction is antigen presentation by immature DCs. Notably, it has been shown that delivering peptides in subimmunogenic forms for a prolonged period of time can result in the induction of CD4+CD25+ Tregs from naïve T cells in peripheral lymphoid organs, even in the absence of a functional thymus (68).

**Treg HOMEOSTASIS**

It was thought that IL-2 is the most important Treg regulator, being required for both Treg maintenance and function (69, 70). More recently, it was shown that Tregs form two distinct populations, the CD44lo CD62Lhi central Tregs, which actively recirculate through lymphoid organs and are sustained by paracrine IL-2, and the CD44hi CD62Llo CCR7lo effector Tregs, which are not found in the lymphoid tissue, do not require IL-2, and are instead maintained by inducible costimulator (ICOS) (71). In vivo, Tregs can proliferate in response to antigens, meaning that Tregs can dynamically respond to their environment (72). It has also been shown that B7/CD28 costimulation plays a critical role in maintenance of Tregs, as shown by experiments reporting a profound decrease in Tregs in B7/CD28-deficient mice (58, 73).

**Tregs IN THE LYMPH NODES**

L-selectin (CD62L) is thought to be crucial for the homing of Tregs to the lymph nodes (LN). CD4+ CD25+ CD62L+ Tregs more potently suppress the proliferative responses of CD25−/− CD4+ T cells than CD4+ CD25+ CD62L−/− Tregs (74). CD62L−/− independent homing induced survival and tolerance in a vascularized cardiac allograft mouse (75). It has therefore been postulated that Treg trafficking to the LNs may be dependent on CD62L (76). A CD69−/−CD25+ CD4+ T-cell subset from the LNs was identified to efficiently suppress CD57+ germinal center (GC)-Th cell-driven B cell production of immunoglobulins. These cells express CCR7 and efficiently migrate in response to CCL19, a chemokine expressed in the T cell zone of LNs. Furthermore, many of these CD69−/−CD25+ CD4+ T-cells populate the T cell rich zone of the LNs; however, some are present in the GCs also (77).

A particular subset of Tregs, the T follicular regulatory cells (Tfr), discovered in 2011, are Tregs that have migrated into the LN, and thus share phenotypes with Tregs, such as FoxP3, CTLA-4 (cytotoxic T-lymphocyte antigen 4), and CD25 expression (78). Additionally, they undergo differentiation and share surface markers with T follicular helper cells (Thf), such PD-1 and ICOS, and important for their localization in the GC s, Bcl-6, CXCR5, and CXCL13 (78). This phenotype allows them to modulate B cell and Thf cell functions in the LN follicles and acts as immune regulators of the GC responses to stimulation (43, 78–80). Currently, CTLA-4 is the only molecule demonstrated to be necessary for full suppression (81, 82); however, IL-10 and TGF-β1 are theorized to play roles in the suppressive function (83). Although a small subpopulation of mature Tfr do not express CD25 (84), depletion of Tfr by anti-CD25 mAb still enhanced humoral responses, with significantly more Ab produced (85, 86).

Soon after their migration to the LNs, Tregs form long-lasting conjugates with DCs. This prevents the DCs from interacting with CD25−/− CD4+ T helper cells (87). Using a murine mathematical model, it has been shown that after occupying the LNs, Tregs do not recirculate, whereas naïve T cells do so readily (88).

**MECHANISMS OF Treg SUPPRESSION**

T regulatory cells produce multiple secretory cytokines that mediate their suppressive activities. The conventional dogma is that cell-to-cell contact is required for the Tregs to exert their suppressive activities (89). However, advances in cell culture capabilities have recently challenged this paradigm. A study using Treg separation from CD4+ T cells with a 0.45-µm permeable membrane demonstrated that, while cell-to-cell contact in the presence of IL-10 and IL-35 appears to indeed be required for Treg activation, the suppressive capabilities of Tregs are not completely mediated by cell contact. Instead, the release of inhibitory factors, such as TGF-β, IL-10, and IL-35, plays a prominent role in Treg-mediated suppression (44, 45).

Transforming growth factor beta-1 is a cytokine secreted by Tregs, which is also present on the cell surface as a membrane bound cytokine. TGF-β1 suppresses non-Treg cells through interactions with the two heterodimer TGF-β receptors, TGF-βRI and TGF-βRII (90, 91). In fact, through the use of T cell-specific Tgfb1 deletion and subsequent Treg cotransfer experiments in Rag1−/− mice, the inhibition of Th1 differentiation and colitis was shown to be dependent upon TGF-β1 production by Tregs (46). Additional studies with TGF-β1 blockades have further supported its role as a mediator of Treg suppressive function (47, 48). TGF-β1 primarily inhibits type 1 T-helper cell (Th1) responses by blocking differentiation through the inhibition of the master regulator T-bet. However, TGF-β1 is also able to directly suppress the effector functions of CD8+ T cells through inhibiting cytokine and effector molecule secretion (49). Beyond direct suppression, TGF-β signaling is important for inducing...
Tregs and HIV Cure Research

Changes in Treg Frequency Throughout HIV/SIV Infection

Treg suppression of the cell-mediated immune response occurs early during the acute HIV/SIV infection, as reported in SIV-infected Rhesus macaques (RMs) (118). In fact, in HIV-infected individuals, the relative frequency of Tregs correlates with the viral load levels and disease progression (119–123), while being inversely correlated with the SIV-specific cytotoxic T lymphocyte (CTL) responses (118). Of note, CD4+ T cell depletion targeting Tregs for death by CD16+ nonclassical macrophages through antibody-dependent cell-mediated cytotoxicity (ADCC) (100).

An indirect Treg suppression mechanism is through consumption of IL-2. CD4+ CD25+ Tregs are able to bind IL-2, preventing non-Tregs from binding and thus inhibiting activation (101), while simultaneously depriving them of the necessary prosurvival signals to prevent apoptosis (102). This competition serves an additional function of enhancing Treg responses by priming them for IL-10 production after TCR stimulation (103, 104).

Extracellular adenosine triphosphate (ATP) is a known inflammatory signal that acts through the P2 purinergic receptors and is released from cells, which have a high intracellular concentration of ATP, during tissue damage [reviewed in Ref. (105)]. Tregs suppress the inflammatory responses to ATP through directly limiting the amount of extracellular ATP by hydrolysis of ATP to adenosine monophosphate (AMP) by CD39, which is highly expressed on the surface of FoxP3+ Tregs (33) and is further upregulated during inflammation (106). Indeed, CD39 plays an important role during HIV infection, as suggested by the observations that CD39+ T cells are inversely correlated with CD4+ T cell counts (107) and polymorphisms that cause decreased expression of CD39 correlate with slower disease progression (107) and decreased suppression of effector T cells (106). Following hydrolysis, AMP is hydrolyzed to adenosine by CD73 on the surface of Tregs (108, 109), which is then shed from the plasma membrane (110). This further increases the suppressive nature of Tregs as adenosine is an anti-inflammatory molecule. In vitro experiments demonstrated that adenosine directly inhibits T cell activation and proliferation through binding to the receptor A2A, preventing TCR-mediated IL-2R (111) and IFN-γ (112) expression. Adenosine further inhibits the Th1 response by decreasing TNF-α and IL-12 production by myeloid dendritic cells (mDCs) while simultaneously increasing IL-10 (113). Additionally, adenosine inhibits IFN-γ and IL-2 production of CD4+ and CD8+ T cells and is inversely correlated to gut inflammation and damage during SIV infection (34). Tregs also suppress T cells through cyclic AMP (cAMP). The binding of adenosine to receptors A2A and A2B induces adenylyl cyclases, increasing the production of intracellular cAMP and suppressing the immune activation [reviewed in Ref. (114)]. Using gap junction inhibitors and cAMP antagonists, it was shown that Tregs transfer cAMP through gap junctions to suppress non-Tregs (115–117).

ROLE OF Tregs IN HIV/SIV INFECTION

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characteristic to acute HIV/SIV infection partially spares Tregs, as suggested by the observation that, in spite of the decrease in their absolute counts, Treg frequency increases during the acute HIV infection (121, 123–125). In HIV-infected subjects receiving ART, Treg frequency is partially restored (120, 126). Interestingly, elite controllers have higher Treg absolute counts, yet lower frequencies, in the peripheral blood and rectal mucosa compared to progressors (119, 127). The mechanism by which Tregs are spared relative to other CD4+ cell subsets during HIV infection remains unclear. In the gut mucosa of SIV-infected RMs, non-Tregs were shown to have significantly higher apoptotic gene expression than Tregs, of which some were apoptotic genes associated with HIV production, supporting the concept that Tregs are relatively resistant to cell death mediated by SIV infection (128). This relative Treg resistance to HIV/SIV infection is further supported by the observations that the number of infected Tregs is similar to CD4+ CD25+Foxp3 cells (129) and that HIV gp120 binding to CD4 enhances Treg survival (122, 130).

Alternatively, the increases in Treg frequency may also be explained by increased conversion of peripheral conventional CD4+ T cells to a Treg phenotype. Ex vivo work performed with plasmacytoid dendritic cells (pDCs) and conventional CD4+ T cells from HIV-infected individuals demonstrated enhanced induction of Treg differentiation when pDCs were stimulated with HIV (131). Similarly, tissue mDCs from SIV-infected NHPs were more efficient at converting non-Tregs to Tregs (132). Increases in the levels of TGF-β characteristic to pathogenic SIV infection of RMs (133) further substantiate the theory of increased pTreg conversion during HIV/SIV infection. This finding is important, because in progressors, Treg suppressive capacity is maintained throughout infection (134), with enhanced function in the LNs, where there is a HIV/SIV reservoir of importance, compared to the peripheral blood (135–137).

In the LNs, Tfr contribute to the impairment of Tfh function (138, 139). Tfh expand during HIV infection (140–142). Their increase is associated with B cell dysfunction (143), as documented by hypergammaglobulinemia, increased GC B cells, and decreased memory B cells (140–142, 144), a likely consequence of hyperactivation through chronic antigenic stimulation (144) and increased cytokine production (141, 142). During HIV infection, the Tfh/Tfr ratio increases and is associated with impaired somatic hypermutation and affinity maturation. These functions can be restored upon Tfr reconstitution (145). While the frequency of Tfr relative to total CD4+ T cells increases during chronic SIV infection, the Tfr fraction of Th is decreased during both acute and chronic stages. Loss of Tfh proliferation control by Tfr during HIV/SIV infection has been examined as a possible explanation for Tfh expansion and may help explain the hyperactivation in the B cell follicles (146, 147). Other studies, however, reported opposite findings, showing that the Tfr/Tfh ratio increases postinfection and through expansion of the regulatory phenotype (139). These data are consistent with Tfh impairment, notably downregulation of ICOS and decreased expression of IL-21 and IL-4 (139), implicating Tfh inhibition in the aberrant humoral response. Thus, whether therapeutic targeting of Tfr in HIV/SIV infection is beneficial or detrimental is still up for debate, although their permissiveness to infection enhances the beneficial aspects of targeting Tfr (148).

**Treg Suppression in HIV Infection**

T regulatory cells are considered both beneficial and detrimental during acute HIV infection (Figure 1). Increased immune activation is a hallmark of HIV infection, and Tregs have been shown to control the activation status of HIV-infected CD4+ T cells (118, 149–152). In the nonpathogenic SIV infection of African green monkeys, an increase in Tregs occurs early during the acute infection, as early as 24 h postinfection, concomitant with significant increases in TGF-β and IL-10 (153). In contrast, during the acute pathogenic SIV infection of RMs, there is only modest TGF-β induction and delayed increases in IL-10 (153). Additional support for the role of Tregs in acute infection was obtained by Cecchinato et al. who showed that CTLA-4 blockade early during the acute infection was detrimental to RMs, resulting in increased viral replication and decreased responsiveness to ART (154). These results may be due to essentially “adding fuel to the fire” by increasing immune activation and consequently expanding the target pool for SIV, although some of this may be due to non-Treg effects. In vitro studies have shown that, in addition to limiting the amount of susceptible cells, Tregs can limit the infection of conventional CD4+ T cells through DC-CD4+ T cell immunological synapses (117). Therefore, Tregs can help in preventing the deleterious pathogenic consequences of HIV/SIV infections by controlling the immune activation status of virus producing cells by shifting them into resting state. This thereby suppresses viral production, and prevents the spread of infection. The corollary of this paradigm is, however, that, by pushing infected T cells into a resting state, Tregs are promoting the generation of the latent HIV/SIV reservoir, the seeding of which starts as early as 3 days post-infection (155), which represents the ultimate obstacle for HIV cure research.

Meanwhile, Tregs also suppress the HIV-specific CD8+ T cell responses (156), their frequency being inversely correlated with the SIV-specific CTL response (118) and T-cell activation (125). The disruption of the cell-mediated immune response against HIV by Treg-mediated suppression of CD8+ T cytotoxic lymphocytes is therefore inhibiting viral clearance in infected individuals, likely leading to an increased viral replication. In fact, in controllers with the protective HLA-B*27 and HLA-B*57 alleles, the HLA-B*27 and HLA-B*57-restricted CD8+ T cells are not suppressed by Tregs, whereas the CD8+ T cells with nonprotective HLA alleles are highly suppressed ex vivo, substantiating the role CD8+ T cells in controlling virus (157). Jiang et al. infected humanized mice with HIV following Treg depletion and compared them with those that were not depleted of Tregs. They found that the Treg-depleted mice had lower levels of infection, as measured by peak viral loads and p24 intracellular staining in plasma and lymphoid tissues, such as spleen and mesenteric LNs (158), supporting a negative effect of Tregs on disease.

Of note, conflicting data were published with regard to the effect of Treg infection with HIV on their suppressive capabilities. Purified bulk Treg populations from HIV-infected individuals retain their suppressive activity (125, 149, 159). In vitro, HIV
infection of Tregs has been reported to either have no effect on Treg functionality (160) or result in loss of functionality (161). However, when functionality was assessed on a per-cell basis, infected Tregs had a lower suppressive capacity and corresponding decreases in genes relating to suppressive function and increases in inhibitory genes compared to noninfected Tregs.
(162), and this altered suppressive capacity may further contribute to the generalized immune activation in chronic infection.

**Tregs AS AN HIV/SIV RESERVOIR**

Suppression of the immune responses is not the only way that Tregs contribute to HIV disease. Treg infection with both SIV (118, 128) and HIV (160) occurs both in vitro (163) and in vivo. Indeed, when naïve T cells were transduced to express FoxP3, their susceptibility to HIV infection increased, and they produced infectious virus at levels comparable to memory T cells (163). In SIV-infected macaques, the fraction of mucosal Tregs containing SIV DNA is higher than that of the non-Tregs, but Tregs harbor less SIV RNA, which was interpreted as an indication that they are less susceptible to productive infection (128). Additionally, mucosal Tregs have a better survival rate than the non-Tregs, supporting increased infection rates without active replication (128). Similarly, in HIV-infected individuals, a larger proportion of Tregs contains HIV DNA than the non-Tregs and importantly, treatment with the histone deacetylase inhibitor (HDACi) valproic acid was able to reverse latency in resting Tregs from more patients than treatment of non-Tregs (164). Y et, (HDACi) valproic acid was able to reverse latency in resting Tregs (164). Y et, (HDACi) valproic acid was able to reverse latency in resting Tregs from more patients than treatment of non-Tregs (164). Yet, when the comparisons are refined by comparing Tregs (CD25+ Tregs from more patients than treatment of non-Tregs (164). Y et, (HDACi) valproic acid was able to reverse latency in resting Tregs from more patients than treatment of non-Tregs (164). Additionally, mucosal Tregs have a better survival rate than the non-Tregs, supporting increased infection rates without active replication (128). Similarly, in HIV-infected individuals, a larger proportion of Tregs contains HIV DNA than the non-Tregs and importantly, treatment with the histone deacetylase inhibitor (HDACi) valproic acid was able to reverse latency in resting Tregs from more patients than treatment of non-Tregs (164). Y et, (HDACi) valproic acid was able to reverse latency in resting Tregs from more patients than treatment of non-Tregs (164). Additionally, mucosal Tregs have a better survival rate than the non-Tregs, supporting increased infection rates without active replication (128). Similarly, in HIV-infected individuals, a larger proportion of Tregs contains HIV DNA than the non-Tregs and importantly, treatment with the histone deacetylase inhibitor (HDACi) valproic acid was able to reverse latency in resting Tregs from more patients than treatment of non-Tregs (164).

**TARGETING Tregs AS A CURE RESEARCH STRATEGY**

The suppressive function of Tregs during HIV infection has opened the forum to assess the benefit of manipulating Tregs for the HIV-infected subjects. However, this is not without its issues, as Tregs are also beneficial in some ways, particularly in suppressing general immune activation. The major problem with targeting Tregs is that the most typical marker for Tregs, the FoxP3 molecule is intracellular and, as such, it cannot be directly targeted. Multiple other targets have however opened the forum to assess the benefit of manipulating Tregs (163) and HIV reservoirs, these data point to a key role for Tregs as targets in cure research strategies.

**TABLE 2 | Strategies for targeting T regulatory cells (Tregs) and/or their function.**

| Impact on Tregs | Target Drug | Rationale | Expected Treg depletion/blockade | Reference | Potential complications |
|-----------------|-------------|-----------|---------------------------------|-----------|------------------------|
| Depletion       | CD25        | IL-2 immunoxygen toxin Treg targeting through attachment to CD25, the receptor for IL-2 and function | Up to 75% depletion of circulating Tregs | (158, 167, 168) | Autoimmunity, toxicity |
| Daclizumab      |             | Prevents IL-2 binding and action, IL-2 is required for maintenance of Treg counts and function | Up to 50% depletion of circulating Tregs | (169–172) |                       |
| CCR4            | CCR4        | Targets Tregs by attaching to CCR4 (effector Treg marker) and causing antibody-dependent, cell-mediated cytotoxicity | Up to 80% depletion of circulating Tregs | (175, 176) |                       |
| Mogalizumab     |             |          |                                 |           |                       |
| Cyclophosphamide| CTLA-4      | Binds to CTLA-4 on Tregs, blocking it from inhibiting lymphocytes | Up to 75% decrease of circulating Tregs | (100, 154, 179, 180) | Autoimmunity, toxicity |
| Functional      | Ipilimumab  |           |                                 |           |                       |
| blockade        |             |           |                                 |           |                       |
| IDO             | 1-methylc-tryptophan | Inhibits IDO, blocking suppressive function | Increased expression of IFN-γ by the lymphocytes in the LNs, decreased plasma viral loads | (181, 182) | Autoimmunity |
Targeting CD25 for Treg Depletion
Two drugs targeting Treg through their constitutive expression of CD25, Daclizumab and Ontak, have been used to deplete them (183, 184). Both compounds bind CD25, but act differently. For Denileukin difitox (ONTAK), which is IL-2 coupled with diphtheria toxin, the IL-2 identifies and binds the CD25+ cells, allowing the diphtheria toxin to enter the cell and cause cell death by ADP-ribosylating host eEF-2 and preventing protein synthesis (185). Ontak has been used to treat CD25+ cutaneous T cell lymphoma (186). Ontak has also been tested with relatively positive results in peripheral T-cell lymphoma, metastatic renal cell carcinoma, and unresectable stage IV melanoma (187–189). Other studies of Ontak administration to melanoma patients, together with a DC vaccine reported no peripheral Treg depletion; yet, this result may be due to the use of a very low dose (190). In the same study, in vitro assessments showed that, while the internalization of Ontak was observable in activated Tregs even at low concentrations, Ontak internalization in resting Tregs only occurred at very high concentrations (190). This may be a potential barrier to the use of Ontak to target the resting reservoir.

The second component, Daclizumab, is a monoclonal antibody to CD25, which prevents the interaction of IL-2 with its receptor. As such, Daclizumab may be used for Treg depletion, as IL-2 is essential for Treg development, maintenance, and function, as discussed above. It has been approved for the treatment of relapsing forms of multiple sclerosis (169, 170) and in adult T cell leukemia to induce remission (191). Daclizumab was also used in radio-immunotherapeutic approaches, after linking it with 90Y, and its administration extended the length of remission in patients with adult T cell leukemia (171). This conjugated Daclizumab was also tested in other CD25+ malignancies, with promising results, especially for patients with Hodgkin’s disease (192). Finally, for a short time, it was used to prevent acute rejection in patients with kidney transplants (193).

In the context of HIV infection, these compounds look promising, with regards to reservoir control. Ontak administration to DKO-hu HSC mice, followed by infection with HIV-RA3, reduced the levels of Tregs in blood, spleen, and mesenteric LN and increased the expression of HLA-DR, a marker of immune activation, on CD4+ and CD8+ T cells. It nevertheless resulted in lower levels of HIV-1 present in the plasma and the lymphoid organs, during the acute stage of infection (158). Furthermore, Ontak administration to humanized NRG mice infected with HIV-1 and completely virologically suppressed by ART, resulted in viral reactivation in spleen and bone marrow. Cell-associated viral DNA levels did not change, indicating that the virions relapsed from the reservoir. The mice were maintained on ART, which prevented the reactivated virus to reinfect cells and, after virus control was achieved post-Ontak administration, the levels of cell-associated viral DNA were significantly decreased in the lymphoid tissue as compared to controls, with no significant change in total CD4+ T cells in the spleen and bone marrow (194).

Ontak administration to chronically SIVsab-infected African green monkeys, resulted in a significant Treg depletion and induced significant CD4+ and CD8+ T cell activation (167). Finally, Ontak administration to SIVsab-infected RMs, a model of spontaneous complete control of HIV infection (195, 196), resulted in the depletion of 75–85% of the peripheral Tregs, an 8- to 10-fold increase in immune activation of the peripheral CD4+ and CD8+ T cells and a boost of SIV-specific T cells (168). Furthermore, a relatively robust virus reactivation was observed, with plasma viral loads reaching up to 10^7 viral RNA copies/mL (from below 5 copies/mL before treatment).

These results suggested that Treg depletion is a plausible strategy for reducing the HIV reservoir in circulation and lymphoid tissues, while boosting effective cell-mediated immune responses (168). Ontak was discontinued for clinical use due to the production issues related to difficulties in the purification from the bacterial expression system. Daclizumab has also been discontinued. However, a new bivalent IL-2 immunotoxin was developed that showed increased potency when compared to the Ontak-like monovalent version (197). When it was used in human CD25+ HUT102/6TG tumor-bearing NSG mouse model, this bivalent immunotoxin was shown to significantly prolong survival of the mice in a dose-dependent manner (198).

Targeting CCR4 for Treg Depletion
T regulatory cells (Tregs) express a high level of CCR4 (199–201), which is the receptor for CC chemokines (MIP-1, RANTES, TARC, and MCP-1) and has been shown to be a coreceptor for HIV-1 (202). Wang et al. developed a diphtheria-toxin based anti-human CCR4 immunotoxin, which effectively binds to and cause protein synthesis inhibition in target cells. It prolongs the survival of tumor-bearing NOD/SCID IL-2 receptor y−/− (NSG) mice injected with human CCR4+ acute lymphoblastic leukemia cells, indicating the efficacy of this drug (173). When the drug was tested in NHPs, it depleted ~80% of CCR4+ FoxP3+ and 40% of FoxP3+ CD4+ T cells in the peripheral blood. In the LNs, although there was a decrease of ~90% of CCR4+ FoxP3+ Tregs, overall FoxP3+ CD4+ T cells were decreased by only 9–22% (174). The anti-CCR4 monoclonal antibody, Mogamulizumab, also shows promise in treatment of peripheral T-cell lymphoma and cutaneous T-cell lymphomas like mycosis fungoides and Sezary syndrome, by depleting CCR4+ malignant cells and CCR4+ Tregs (175, 176).

Cyclophosphamide
Cyclophosphamide (Cy) is a well-established chemotherapeutic agent, which is widely used for the treatment of leukemias and lymphomas. In high doses, Cy acts as a nonselective cytoreductive agent, which directed its uses as part of a preparation regimen for allogeneic stem-cell transplantation (203) and in treatments for systemic lupus erythematosus (SLE) (204–206). In low, metronomic dosages, Cy retains its antitumor capabilities, with reduced side effects and improved clinical responses (207). In mice, low-dose Cy administration selectively and significantly depleted and reduced the functionality of Tregs (208, 209). In vitro, CTLs and T helper cells are more resistant to Cy cytotoxicity than Tregs (210). Treg selectivity has been attributed to decreased DNA repair, as demonstrated by the increased and sustained DNA intercross-linking, as well as increased and sustained phosphorylated histone 2AX. A different mechanism for sensitivity, decreased production of glutathione, a detoxifier
for Cy and its active metabolites, was also evoked. Indeed, Tregs have decreased ATP levels, which abrogate glutathione production, thereby inducing hypersensitivity to Cy (211). Interestingly, CCR2+ Tregs are preferentially depleted in mice over CCR2− Tregs. An analysis of the cell cycles demonstrated increased proliferation and activation in CCR2+ Tregs (212).

Patients treated with a single dose of 300 mg/m² Cy experienced a ~20% Treg decrease sustained for 25 days and a decrease in proliferation marker Ki-67, further substantiating the loss of Treg homeostatic proliferation (177). Further studies of Cy in humans showed that end-stage cancer patients treated with metronomic dosing of 100 mg of Cy per day for 7 days for 4 weeks of on/off, such that the cumulative dose of ~777 mg/m² was split between 2 weeks with a week without treatment in between, had greater than 50% decrease in both relative frequency and absolute counts of Tregs at day 30 of treatment. Importantly, treatment caused an increase in CD8+ T cell and NK cell cytotoxicity, a requirement for adequate clearance of infected cells during HIV cure approaches. Interestingly, when the dose was increased to 200 mg/day, the selective depletion of Tregs was ablated, underpinning the importance of the low dose for specific Treg targeting (178).

To be an effective therapy for HIV, Cy must be effective in depleting Tregs from the LNs, where there is a major viral reservoir. A study in mice showed that Cy treatment was beneficial in the LNs by selectively depleting the CD8+ lymphoid-resident DCs while sparing the skin-derived migratory DCs and pDCs in the LNs and spleen. This selective depletion in turn boosted antigen presentation and cytokine secretion by the mDCs and pDCs, with a reduction in Treg suppressive capabilities. These results were confirmed by an adoptive transfer of CD8+ DCs, which abrogated the immune enhancement (213). When patients were treated with a single IV low-dose of 300 mg/m² Cy, a less immunosuppressive environment compared to controls was observed in the LNs, including significant decreases in IL-10, IL-6, and VEGF (214). Altogether, these results demonstrate that Cy administration is effective in modulating Tregs from both the LNs and periphery.

Little is known about Cy as a therapeutic approach for HIV cure. In an HIV-positive patient with SLE, treatment with Cy induced an enormous burst in viral replication, with plasma viral loads peaking to >1.3 × 10⁷ copies/mL and quickly returned to below detectable levels (215). Using escalating doses up to 1.6 g/m², Bartlett et al., monitored the effects of Cy on HIV DNA in LNs and PBMCs and plasma viral loads. They found no significant difference in the HIV DNA burden of LNs and PBMCs versus the control group, but, of note, plasma viral loads were not suppressed in these patients, with two subjects out of five admitting to nonadherence to ART (216). Thus, it is possible that the increase in plasma viral loads and lack of viral DNA clearance may have been due to nonadherence.

Based on these data and the impressive benefits of Cy during various cancer treatments, low-dose Cy could be an effective therapy to decrease HIV reservoir, through its Treg-depleting effect. However, further studies are necessary to detail the potential of Cy to enhance HIV-specific CTL responses and/or reactivate latent HIV.

**THERAPIES TARGETING Treg FUNCTION**

Various therapies to affect Treg function have also been tested. CTLA-4+PD-1m CD4+ T cells from multiple tissues are enriched for replication-competent SIV in infected RMs under ART, suggesting a potential therapeutic target for reservoir elimination (217). During HIV infection, CTLA-4 plays a role in suppression of HIV-specific T cells, with CTLA-4 blockade enhancing CD4+ T cell functionality, i.e., IFN-γ production and cell proliferation (218, 219). In an HIV-infected individual treated with ipilimumab (α-CTLA-4 mAb) for melanoma, viral plasma loads remained below the limit of detection using standard qPCR methods, whereas a general decline in plasma viral loads was seen when using the single copy assay, with an opposing increase in cell-associated unspliced RNA post-treatment, likely due to expansion of infected T cells (179). Additionally, in chronically infected RM given blocking CTLA-4 Ab while on ART, decreases in viral RNA was noted when therapy was interrupted, along with an increase in the SIV-specific immune response (180). However, when the same blockade was used in early infection with the pathogenic SIVmac251-infected RM model, it increased immune activation, viral replication, but did not augment SIV-specific responses, and abrogated responsiveness to ART (154). Thus, further studies need to be conducted to determine whether stand-alone CTLA-4 blockade can be used as a latency reversing strategy.

As mentioned earlier, Tregs also express PD-1, which affected their homeostasis (17, 220). PD-1 also is thought to participate in Treg suppression (221). Due to its major role in contribution to T cell exhaustion (222, 223), efficiency of PD-1/PD-L1 blockade is widely studied in HIV-1 infection. However, the lack of specificity to Tregs of these interventions puts PD-1 targeting out of the scope of this review [reviewed in Ref. (224)].

Indoleamine 2,3-dioxygenase has been observed to increase during HIV infection and may suppress the antiviral responses (225). Thus, IDO blockade has been attempted to enhance the antiviral response to HIV/SIV (181, 182). In SIV-infected macaques under ART, treatment with the IDO inhibitor 1-methyl-d-tryptophan (d-1mT) reduced plasma viral loads and SIV RNA in LNs (181). 1-mT combined with CTLA-4 blockade in SIVmac251-infected macaques under ART did not provide better control of viremia (182). Additionally, this treatment induced acute pancreatitis in all animals, whereas the same ART regimen given alone induced pancreatitis in only 10–20% of the animals (182). These data suggest either an exacerbation of ART toxicity, or more likely, the induction of auto-immune responses against pancreatic antigens. Whatever the underlying mechanisms, such findings are a cautionary warning of the potential risk of any Treg manipulation in vivo.

**CONCLUSION**

Treg suppression of virus-specific immune responses may limit the efficacy of virus reactivation strategies, which require effective killing of the reactivated HIV/SIV reservoir. As a result, Tregs may play a central role in shaping the HIV reservoir and compromising the HIV/SIV-specific immune responses. Future research should
focus on further refine the effects of various Treg manipulation techniques on the reservoir. Here, we have described several promising Treg treatments that may either suppress Treg activity or kill Tregs altogether. Treg depletion, which has the ability to directly target a small fraction of the reservoir, reactivates the virus, and boost cell-mediated immune responses, might be a desirable strategy for cure research. Although standalone Treg manipulations are promising, they can quite easily be added to other regimens. In the future, investigations into combining Treg therapies with the more traditional viral reactivation therapies, i.e., HDACis, PKA agonists, etc., or vaccinations may prove to be valid cure strategies.

**AUTHOR CONTRIBUTIONS**

Outlined the manuscript (AK, RS, IP, CC, CA); drafted the manuscript (AK, RS); reviewed the manuscript (IP, CC, CA); Outlined the manuscript (AK, RS, IP, CC, CA, CC); drafted the figure (RS, AK).

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