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Review

Mechanisms Underlying the Induction of Regulatory T cells and Its Relevance in the Adaptive Immune Response in Parasitic Infections

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Abstract

To fulfill its function, the immune system must detect and interpret a wide variety of signals and adjust the magnitude, duration, and specific traits of each response during the complex host-parasite relationships in parasitic infections. Inflammation must be tightly regulated since uncontrolled inflammation may be as destructive as the triggering stimulus and leads to immune-mediated tissue injury.

During recent years, increasing evidence points to regulatory T cells (Tregs) as key anti-inflammatory cells, critically involved in limiting the inflammatory response. Herein, we review the published information on the induction of Tregs and summarize the most recent findings on Treg generation in parasitic diseases.

Key words: regulatory T cells; parasitic infections; inflammation, dendritic cell; regulation; immune response; cytokines; malaria; Leishmania; *Trypanosoma*; *Schistosoma*, nematodes.

Regulatory T cells: phenotypes and induction

Regulatory T cells (Tregs) play an important role in the control of the immune response. Different types of Treg cells have been described, and may be classified into two main groups: thymic and inducible. Thymic or natural Treg cells (nTregs), are produced in the thymus and are present in the host's bloodstream before pathogen or damage exposure. Inducible Treg cells (iTregs) are those cells that acquire a regulatory function in the context of a given infection or a neoplastic process. Inducible Treg cell populations include: T regulatory 1 (Tr1) cells, which secrete IL-10; T helper 3 (Th3) cells, which secrete TGF-β, and converted Foxp3+ Treg cells [1-3]. This review is focused on the induction of CD4+ Tregs, mostly by Foxp3+ expression, and on their suppressor activities during parasite infections.

Inducible Treg cells are generated in the periphery and exert their suppressor activity mainly by producing IL-10, IL-35, and TGF-β [4, 5]. Initially, these iTreg cells are conventional T cells expressing low or null levels of CD25 (CD25low/-) [6]. CD25 expression could be up-regulated according to environmental conditions [7]. On the other side, Foxp3 expression is up-regulated by signaling pathways initiated by T cell receptor (TCR), co-stimulatory molecules, IL-2R, programmed death ligand 1 (PDL1), transforming growth factor-β (TGF-β) receptor, and Notch [8-10]. In fact, the TCR density on the cell surface and its affinity to the antigen play a key role in

Treg induction. It has been demonstrated that TCR stimulation by a strong agonist in low dose resulted in maximal induction of Foxp3 *in vivo*. Also, the density and duration of TCR interactions define a cumulative TCR stimulation that determines initial Foxp3 induction [11]. In contrast, high doses of TCR engagement in presence of CD28 co-stimulation induce NF-κB signaling, which prevents Foxp3 induction in mice [12]. On the other hand, recently it has been demonstrated that CD28 signals alone, independently of TCR-mediated stimulatory pathways, are sufficient to induce Foxp3 transcription in CD4(+)CD25(-) T cells. Additionally, the stimulation of CD28 associated with TCR can regulate Foxp3 expression; the authors propose that, under these conditions, CD28 signals mediate Foxp3 trans-activation by nuclear translocation of RelA/NF-κB [13, 14]. Furthermore, the presence of regulatory cytokines like IL-10, TGF-β, and IL-35 during the priming of T lymphocytes favors regulatory T cell generation, function and maintenance (this point is discussed later). Most probably, the accumulation of signals that yield a regulatory T cell phenotype depend on the molecules expressed on the cell surface, their interaction with environmental molecules, and the prevalent intracellular signaling. All of these factors may in turn be modified by infective agents as a way to evade immunity.

In that sense, several mechanisms have been reported as involved in Treg-mediated immune suppression, offering a wide range of possibilities to exhibit their function under different environmental and requirement conditions. Some of these mechanisms are reviewed elsewhere [15].

Role of dendritic cells and cytokines in Treg induction during the adaptive immune response

Tregs can be generated by conversion from naïve T cells or by expanding the population of pre-existing Treg cells. The nTregs antigen-specificity issue remains controversial since they are expanded from pre-existing Tregs. In contrast, Tregs induced as a consequence of adaptive immune response are generally antigen-specific [16]; however, Treg cells can inhibit responder T cells of unrelated antigen specificity [17]. On the other hand, the Treg induction pathway depends on the interaction of T cells with tolerogenic dendritic cells, anti-inflammatory cytokines, glucocorticoids, vitamins, or other suppressive molecules produced by pathogens [18].

Role of dendritic cells

The immunological paradigm places fully mature dendritic cells (DCs) as immunity inducers. It is currently believed that DCs integrate a variety of incoming signals and decide whether protective immunity or tolerance develops. Mature DCs might be crucially involved in the expansion and induction of Treg cells. In fact, conventional CD4+ cells become either specific T helper or T regulatory cell subsets depending upon the affinity of their TCR to the antigen, the strength of the co-stimulatory signals provided by antigen-presenting cells (APCs), and the cytokine milieu (Fig. 1).

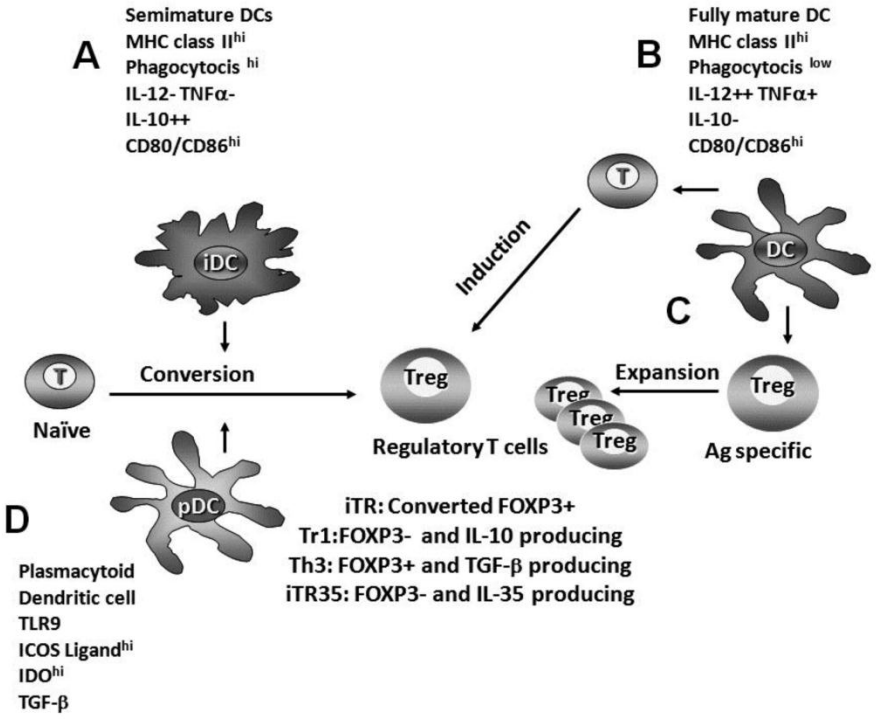

Figure 1. Different pathways of regulatory T cell induction. A) Semimature dendritic cells promote the conversion of naïve T cells into Treg cells. B) Fully mature dendritic cells generate new antigen-specific regulatory T cells during immune response priming. C) Fully mature DCs may be crucially involved in the expansion of preformed Treg cells. D) Plasmacytoid dendritic cells also promote Treg cell induction by ICOS-L, TLR9, TGF-β and IDO-dependent pathways.

In contrast, semimature DCs promote T cell tolerance by the conversion of naïve T cells into Treg cells. The absence of inflammation arrests dendritic cells into a semimature state (iDC). iDCs are characterized by the expression of MHC II, a high phagocytosis capacity, and low CD80/CD86 expression; they produce IL-10, but neither IL-12 nor TNFα. The inability of producing IL-12p70 in bioactive amounts together with IL-10 generates a unique phenotype of semimature DC that induces Treg cells (Fig. 1) [19]. In turn, these newly formed Treg cells induce a suppressive environment.

Role of cytokines

IL-10

IL-10 plays a role in Treg increase and maintenance [20]. The role of IL-10 in regulatory T cell induction probably involves several events: in APC, IL-10 reduces antigen presentation by trapping peptide-loaded major histocompatibility complex type II (MHCII) molecules, reducing the co-stimulatory molecules CD80/CD86 expression, and destabilizes cytokine mRNAs. Additionally, it has been reported that high levels of CD40, CD86, and PD-L1 in APC favor *de novo* induction of Tr1 cells [21].

In turn, IL-10 conditions CD4+ T cells to become unresponsive to antigens, and these cells lose the capacity to produce cytokines, probably by induction of suppressors of cytokine signaling (SOCS-1 and -3) [22]. Other IL-10 effects on peripheral generation of Treg cells may be mediated by activation of the Notch-dependent signaling pathway. Transgenic mice carrying the constitutively active intracellular domain of Notch3 in thymocytes and T cells fail to develop experimental autoimmune diabetes. The inability to develop the disease is associated with an increase of CD4+CD25+ Treg cells. In fact, an accumulation of Tregs in lymphoid organs and pancreas infiltrates is observed. This accumulation is paralleled with an increased expression of IL-4 and IL-10 [23]. Furthermore, the stimulation of purified murine CD4+ T cells with antigen, anti-CD3 and anti-CD28 antibodies has proved to induce a transient increase in Notch ligand and receptor expression [24]. Probably, Notch has a key role in boosting the differentiation and possibly the function of Treg cells [23]. Likewise, in naïve mouse CD4+ T cells, Notch induces IL-10 production via a signal transducer and activator of transcription 4 (STAT4). IL-10 could act as a positive autocrine factor in the development of IL-10-producing Tregs [25].

An indirect mechanism of action of IL-10 in the generation of suppressor cells occurs through CD4+ anergic induction. In one pathway, IL-10 induces anergy in CD4+ T cells [26]; in another pathway, treatment of dendritic cells with IL-10 yields anergic T lymphocytes [27, 28]. These anergic T cells act as suppressor cells by competing with other antigen-stimulated T cells for the membrane of APCs and for locally produced IL-2 [20].

TGF-β

Evidence of the *in vivo* effect of TGF-β in a Treg cell pool expansion was described in diabetic mice stimulated with TGF-β. In these mice, TGF-β inhibited autoimmune type I diabetes development, in addition to an increase in the Treg frequency in pancreatic intraislets. These cells showed high levels of intracellular CTLA-4 and Foxp3 expression [29]. Early studies in human T cells demonstrated that TGF-β was necessary to induce Tregs. Stimulation of human CD4+ cells with TGF-β increases the number of Tregs and intracellular expression of CD25 and CTLA-4. This expansion was due to both an increased proliferation and the protection of these cells from activation-induced apoptosis [30].

TGF-β promotes the induction of Treg cells accompanied by an increase in Foxp3 expression. In mice, it has been demonstrated that TGF-β is able to convert CD4+CD25-Foxp3- non-Tregs into CD4+CD25+Foxp3+ Tregs. Evidence for the role of TGF-β was collected in Foxp3-mRFP mice, in which Foxp3-expressing cells were marked by messenger red fluorescent protein (mRFP) expression. Upon TCR engagement, TGF-β induced *de novo* Foxp3 expression. Furthermore, only Foxp3+CD4+ cells but not their Foxp3-CD4+ counterparts showed regulatory activity [31]. Also, in an independent study, Zhang *et al.* reported that engagement to TCR and CTLA-4 delivers a specific signal which cooperates with TGF-β signaling molecules to initiate Smad signaling and recruits co-activators to activate Foxp3 expression [8, 32].

Foxp3 regulation and induction mechanisms are currently being elucidated. Fantini *et al.* suggested that TGF-β may induce Foxp3 directly by binding to the inhibitory Smad7 promoter region to turn off its expression. Thus, TGF-β might produce a feedback regulation of TGF-β signaling that may result in a cumulative Foxp3 expression, facilitating the conversion of T cells into Tregs [33].

Foxp3 transcription is a key factor in Treg induction, differentiation, function and survival. *foxp3* gene expression is controlled by a core promoter and at least three distal enhancers. During T cell activation, a number of transcription factors (NFAT, AP1, CREB, SP1, c-Rel, RUNX, TIEG1, RAR Stat5, ATF, and

Smad 3) bind to conserved noncoding sequences (CNS) in the promoter and in intron 2 [34]. In fact, the 5′ and 3′ CNS in intron 2 serve as enhancer sites. The formation of a Foxp3-specific "enhanceosome" containing nuclear factor-kB, c-Rel, NFATc2, p65, Smad3, and CREB as FOXP3 expression inducers [35-38] has been identified. Recent studies indicate that murine TGF-β drives Foxp3 expression, probably in two ways: 1) through the induction of activated Smad3, as it initially binds to an enhancer site in intron 2 of *foxp3* gene and then interacts with nuclear factors to form an "enhanceosome", which binds to the *foxp3* promoter, resulting in Foxp3 transcription; 2) TGF-β induces increased H4 histone acetylation in the region of NFAT/Smad binding; this increases the accessibility of various transcription factors and facilitates the FOXP3 promoter activity [38].

Notably, the relationship between TGF-β and CTLA-4 is essential for Treg induction. Zheng et al. have demonstrated that TGF-β is required to induce Foxp3 expression, but in CTLA-4-/- deficient mice TGF-β stimulation is not able to convert CD4+CD25- cells into Tregs. This group has also demonstrated that CTLA-4 ligation to CD80 shortly after T cell activation enables TGF-β to induce CD4+CD25- cells to expressing FoxP3 and developing suppressor activity. Additionally, Foxp3 has been reported to up-regulate CTLA-4 expression; thus, a TGF-β/CTLA-4/FoxP3/CTLA-4 positive loop may be vital for the induction and maintenance of regulatory T cells [32].

It was suggested that TGF-β alone is unable to mediate Treg cell induction. In fact, the combination of IL-2 and TGF-β play different but complementary roles in Treg induction. In IL-2 KO mice, Foxp3 expression cannot be sustained in the periphery; also, IL-2 KO T cells stimulated with TGF-β are not able to convert CD4+ T cells into Treg cells. Thus, during Treg induction it is likely that TGF-β induces Foxp3 expression in newly induced Tregs, and that Foxp3 stability is apparently IL-2-dependent [39]. In agreement with these observations, Foxp3+ Treg induction requires the expression of IL2R and TGF-β receptors [39, 40]. Additionally, the administration of IL-2 results in the stabilization of Foxp3 expression in TGF-β-induced Tregs in vivo [41]. Additionally, an indirect effect might take place during Treg induction: while IL-2 could promote the growth of these cells, TGF-β would protect them from activation-induced apoptosis [20].

Recently, new molecules have been implied in Foxp3 induction by TGF-β. In mucosal tissue, mature tolerogenic DCs producing retinoic acid also induce Foxp3+ Tregs via a TGF-β-dependent mechanism. In fact, retinoic acid enhances TGF-β signaling by increasing the expression and phosphorylation of Smad3, and this results in increased Foxp3 expression, even in the presence of IL-6 or IL-21 [42].

Furthermore, it has been proposed that Activin A may contribute to the expansion of peripheral Treg cells. Activin A is a pleiotropic TGF-β family member and is expressed in response to inflammatory signals. Huber *et al.* reported that Activin A together with TGF-β1 shows synergistic effects on the Treg conversion rate and seems to be essential for Treg induction [43].

IL-35

IL-35 is a heterodimeric cytokine composed by an Epstein-Barr virus-induced gene 3 (EBI3) subunit plus the p35 subunit of IL-12. IL-35 is a Treg cell-specific cytokine, required for the maximum regulatory activity of mouse Treg cells *in vitro* and *in vivo* [4]. Recently, Collison and colleagues described a newly identified population of CD4+ Treg cells, induced by IL-35, called "iTR35 cells". They have demonstrated that human and mouse CD4+ T cells activated with beads coated with anti-CD3 and anti-CD28 antibodies in the presence of IL-35 substantially up-regulated *EBI3* and *IL12A* mRNA, which encode the two constituents of IL-35. iTR35 did not express Foxp3 or other key suppressive cytokines (IL-10 or TGF-β); thus, the proposed phenotype is CD4+Foxp3−Ebi3+p35+IL-10−TGF-β−. iTR35 mediated suppression exclusively via IL-35 and seemingly independently of IL-10 and TGF-β. iTR35 expresses CTLA-4 in a relatively low percentage (<15%), and its function is not yet clear. Finally, these authors showed the regulatory capacity of iTR35 by restoration of immune homeostasis and preventing autoimmunity in Foxp3−/− mice, preventing EAE, controlling T cell proliferation in a lymphopenic setting, and limiting the pathogenesis during experimental colitis [44].

Role of glucocorticoids in Treg cell induction

Glucocorticoids (GCs) inhibit the ability of APCs to stimulate effector T lymphocytes and favor Treg cell induction. Emilie *et al.* showed that GCs and IL-10 stimulate the production of an intracellular factor called glucocorticoid-induced leucine zipper (GILZ) in monocytes/macrophages and in human DCs [45]. GILZ prevents the expression of activation molecules such as MHC II, CD80, CD86, and CD83, and inflammatory chemokines. Besides, GILZ stimulates the expression of immunoregulatory molecules like B7-H1/CD274, ILT3/CD85k and IL-10 [46]. Hamdi *et al.* showed that GILZ-expressing DCs stimulate in T

lymphocytes the expression of the Tregs markers CD25hi, Foxp3, and CTLA-4. Induced Tregs are able to produce IL-10 and inhibit T CD4+ cell proliferation in an antigen-specific manner. In fact, IL-10 production by Tregs is essential for suppression. The mechanism probably implies two different ways: 1) IL-10 acts as a growth factor for Tregs, and 2) IL-10 contributes to their suppressive functions [47, 48].

The concomitant administration of vitamins and dexamethasone induces differentiation of regulatory T cells from naïve T CD4+ cells in both humans and mice. These Treg cells are characterized by: (a) IL-10 production, (b) attenuated secretion of IL-2 and IFNγ, (c) lack of induction of the Th1 or Th2 phenotype, and (d) significant antigen-specific and non-specific suppression of T cell proliferation. The mechanism described may imply a direct effect on dendritic cells, as mentioned above [49-53].

Another mechanism of Treg cell induction may involve a direct effect of vitamin D3 and dexamethasone on T cells. Barrat *et al.* have shown that vitamin D-Dex treatment induces the development of IL-10-producing T cells in absence of APCs. When naïve CD4 T cells were simultaneously stimulated with vitamin D-Dex, anti-CD3 and anti-CD28, higher numbers of IL-10-producing cells were obtained. The use of anti-IL-10R mAbs inhibits Treg induction in these APC-free systems. Thus, IL-10 acts as a positive autocrine factor in the development of IL-10-producing Treg cells [54]. This effect could be associated to the down-regulation of NF-kB p50 and c-Rel protein expression [50, 54, 55].

Plasmacytoid dendritic cells in Tregs induction

Plasmacytoid DCs (pDCs) play distinct roles in regulating T cell-mediated adaptive immunity. Firstly, pDCs express different PAMS and possess the ability to produce large amounts of type-1 IFNs. Also, maturing pDCs exhibit the ability to generate IL-10-producing Treg cells [56]. These mechanisms are possibly mediated by indoleamine 2,3-dioxygenase (IDO) and inducible T-cell co-stimulator ligand (ICOS-L), among other molecules [56, 57]. pDCs do not express functional IDO constitutively; however, they can be readily activated by tolerogenic ligands or by CpG. In fact, binding of CpG to TLR9 and CTLA-4-Ig or GITR-Ig to B7 in pDCs promotes IDO protein expression. IDO catalyzes the conversion of tryptophan into kynurenine, a critical metabolite involved in the generation of Tregs with potent cell suppressor function from CD4+CD25- T cells [57-59]. Recently it has been demonstrated that IDO acts as an intracellular signal transducer, in response to TGF-β, to induce a stable regulatory phenotype in plasmacytoid dendritic cells. These pDCs are able to generate Tregs from naïve T cells [60].

The role of ICOS-L in Treg generation was elucidated by Ito *et al.*, 2004. They found that naïve peripheral CD4 T cells co-cultured with pDC in the presence of neutralizing antibodies against ICOS-L inhibits IL-10 production by Treg cells, but not other cytokines such as IFN-γ or TNFα. On the other side, in the same study it was shown that the stimulation of naïve CD4 T cells with anti-CD3 alone or anti-CD3 in the presence of ICOS-L generates Treg cells capable of producing high levels of IL-10 but not IL-4 [56]. Furthermore, a specific defect in Treg cell-mediated mucosal tolerance has been observed in ICOS-deficient (ICOS−/−) mice [61]. In agreement with this, ICOS-L has been previously implicated in the generation of IL-10-producing Treg cells, both in human studies and mouse disease models [62, 63].

Moreover, pDC are not a main APC subset and probably this would limit their role in regulatory T cell induction. However, the interaction of TLR9 with CpG favors pDC suppressor activity, as stated above. This may promote a suppressor environment accompanied by Treg induction.

The capacity of parasites to activate TLR9 in pDC and thus induce Treg cells as a part of their control strategies on the host's immune response still needs to be elucidated.

Generation of regulatory T cells in parasitic infections

Regulatory T cells help controlling the immune response, but in some cases pathogens may promote an excessive regulatory control, which allows parasite replication without restraint and compromises the host. The consequence of such control is an enhanced pathogen survival and, in some cases, a long-term pathogen persistence. Even though there are clear evidences of Treg cell induction during many parasitic infections, their role in these parasitoses is not well established yet. Herein we discuss some illustrative examples of parasitic infections that involve Tregs in their pathogenesis.

In leishmaniasis

Parasites of the genus *Leishmania* are the causative agents of cutaneous, mucocutaneous or visceral leishmaniasis [64]. *Leishmania sp.* triggers multiple effects on the functions of macrophages and dendritic cells that promote immunomodulation of innate immune response and impair their capacity to initiate Th1 cell immunity (as discussed in [65]).

It has been suggested that intracellular forms of *Leishmania* (promastigotes and amastigotes) produce yet unknown molecules that promote an immunoregulatory environment, probably through IL-10 and TGF-β. This way, the induction of regulatory T cells permit a low-level persistence of the parasite, which in turn maintains the immunological stimulus (Table 1) [66, 67]. In fact, *in vivo* experiments have suggested that transferred CD25- T cells become CD25+. These Tregs are homed at the parasite inoculation site, where they promote the infection establishment and ensure the long-term survival of the parasite in the host [66]. Actually, CD103 expression has been shown to be essential for Tregs homing. Genetically susceptible BALB/c mice that lack CD103 become resistant to *Leishmania major* infection, a phenotype associated with a poor capacity of Tregs to be retained in the infection site (skin) [68]. Conversely, Treg cells accumulate in the lesions of patients with acute cutaneous leishmaniasis. These Tregs are functionally suppressive, since they decrease the IFN-γ production. The main suppressive action observed in intralesional Tregs implies IL-10 and IDO [69].

Different authors have proposed that, during infection, the parasite promotes Treg induction, which might also modulate effector T cells or antigen-presenting cell (APC) functions by means of cell contact-dependent mechanisms [66, 67, 70]. Other teams have observed that lower CD40 expression levels in APC are related to the degree of infection of *L. donovani*. Interestingly, the parasite down-regulates CD40 expression and up-regulates IL-10 and TGF-β expression in APC during infection. All of these conditions are required for efficient Treg induction [71]. Additionally, *Leishmania* amastigotes can infect dendritic cells and down-regulate early innate signaling events, resulting in impaired DC function that includes lower CD40 and CD83 expression, suppressed IL-12p40, IL-12p70, and IL-6 production, and increased IL-10 production [70].

Recently, it has been reported that *Leishmania major* cutaneous infection stimulated IDO expression in local lymph nodes [72]. The known effect of IDO on dendritic cells suggests that this molecule could be involved in Treg induction during cutaneous infection.

Table 1. Regulatory T cells (Tregs) in leishmaniasis

Parasites	Host	Molecule involved	Cells involved	Role of Tregs in pathogenesis	Ref #
L. major	Mouse	IL-10	*Tr1	Tregs accumulation favors parasite persistence	[66, 134]
L. donovani	Mouse	TGF-β	º Th3	Enhance parasite growth and exacerbate infection	[135]
L. donovani	Human	IL-10	Tr1	Enhance skin lesion	[136]
L. guyanensis	Human	IL-10, IDO	‡Tregs	Accumulation in lesions from acute cutaneous leishmaniasis; Tregs contribute to infection chronicity	[69, 137]
L. infantum	Mouse	IL-10, TGF-β	Tr1, Th3	Influence local immune response that favors parasite persistence	[138]
L. braziliensis	Mouse/Human	IL-10, TGF-β	Tregs	Enhance cutaneous lesions	[139, 140]
L. braziliensis	Macaque	IL-10	Tregs	Regulate local immune response	[141]

*Tr1: IL-10 producing cells, ºTh3:TGF-β producing cells, ‡Tregs: conventional Foxp3+ cells

In schistosomiasis

Schistosomiasis is one of the most common parasitic infections worldwide. Upon infection, adult parasites migrate to the mesenteric veins, where they lay hundreds of eggs per day. Some of these eggs are trapped in the liver, gut and other organs, causing a vigorous granulomatose response [73]. The promotion of Tregs could be regarded either as beneficial or detrimental for the parasite. This approach to the role of Treg cells in schistosomiasis has not been explored yet, and deserves further research. We propose two hypothetical possibilities about the role of Tregs in schistosomiasis. In one, the immune system itself induces Tregs to control the exacerbated immune response that produce granulomas. Thus, granulomas would be useful to eliminate the parasite, since they would help locating the eggs and triggering the immune response. As a second possibility, parasite antigens mediate Treg induction during granuloma formation. The fibrosis promoted by collagen production induced by IL-13 [73, 74] supports the first possibility. IL-13 could also be directly involved in the induction of Treg cells. The engagement of the IL-4R alpha-chain by binding cytokines IL-4 and IL-13 was identified as an inducer of CD25+ Tregs from peripheral naïve CD25-CD4 T cells [75]. Nevertheless, it is not known if this IL-13 production during schistosomiasis infection is related or not to Treg cell generation. Supporting the second possibility, Treg cells have been associated to egg antigens during *Schisto-*

some infections in mice [76, 77]. In fact, IL-10-secreting CD4+CD25+ cells isolated from granuloma from chronically infected mice can suppress the proliferation of CD4+ T cells [78, 79]. An alternative approach to address the role of Treg cells in granulomas was adopted by Singh *et al.*, 2005, using experimentally infected mice with *Schistosoma mansoni*. They found that granulomatous livers at 8 and 16 weeks after infection showed 10- and 30-fold increases in Foxp3 expression compared with normal liver. Also, the percentage of Treg cells in granuloma rose from 12% at 8 weeks to 88% at 16 weeks after infection. Retroviral transfer of the Foxp3 gene at the onset of granuloma formation enhanced Foxp3 expression fourfold in granuloma CD4(+) CD25(+) T cells, and strongly suppressed full granuloma development [80]. All these data suggest that *Schistosoma* may be implicated in the evolution of granulomas (Table 2).

In trypanosomiasis

Trypanosomes are zooflagellate protozoans that cause persistent infections accompanied by a profound immunosuppression [81]. *Trypanosoma cruzi* is an intracellular parasite, primarily located in the tissues, while African trypanosomes are extracellular forms circulating in the blood. The role of Tregs in trypanosomiasis infection is controversial. On one side, African trypanosomiasis is characterized by gradual expansions of the total number of Tregs, starting at parasite establishment and continuing during the chronic stages of the disease, which favors parasite tolerance (Table 3) [82, 83]. On the other side, Tregs have a limited role on *Trypanosoma cruzi* pathogenesis [84, 85]. In fact, depletion of Treg cells during acute and chronic infection neither improved nor worsened the course of the infection and the immunity involved [86]. However, it is important to note that *T. cruzi* and African trypanosomes are completely different, and that immune response induced by an extracellular or by an intracellular parasite will trigger different effector mechanisms. Thus, the role of Tregs in trypanosomiasis may depend on factors such as the parasite biology, the environment, and the host immune response.

Table 2. Regulatory T cells (Tregs) in schistosomiasis

Parasites	Host	Molecule involved	Cells involved	Role of Tregs in pathogenesis	Ref #
S. mansoni	Mouse	IL-10	*Tr1, ‡Tregs	Suppress Th1 and Th2 response and increase the susceptibility to infection	[78, 79]
S. mansoni	Mouse		Tregs	Regulate immune response, favoring eggs survival	[133]
S. japonicum	Mouse	IL-10, TGF-β	Tregs	Regulate immune response, favoring parasite survival	[142]
S. mansoni	Mouse	CD103, GITR, OX40, CTLA-4	Tregs	Limit and control granuloma growth	[143]
S. japonicum	Mouse	IL-10	Tregs	Promote worm burden	[144]

*Tr1: IL-10 producing cells, ‡Tregs: conventional Foxp3+ cells

Table 3. Regulatory T cells (Tregs) in trypanosomiasis

Parasites	Host	Molecule involved	Cells involved	Role of Tregs in pathogenesis	Ref #
T. congolese	Mouse	IL-10	‡Tregs, *Tr1, ∞nTregs	Down-regulate the Th1 response and preserve host parasite clearance	[83]
T. cruzi	Human	IL-10, CTLA-4	Tregs,Tr1	Control early stages and favor the disease progression	[145]
T. congolese	Mouse		Tregs	Prevent early protective response	[82]
T. cruzi	Mouse	GITR	Tregs	Control inflammation	[85, 140]
T. cruzi	Mouse		Tregs	Neither improve nor worsen the outcome of immune response	[86]

*Tr1: IL-10 producing cells, ‡Tregs: conventional Foxp3+ cells, ∞nTregs: Natural Tregs

On the other hand, Treg generation during trypanosome infection is supported by multiple evidences. The mechanism may imply a default in DC activation or a specific cytokine balance to an immunosuppressive environment [87]. At early stages of infection, Guilliams *et al.*, 2007, observed that Treg population is expanded in spleen and liver of mice infected by *T. congolense*. These cells produced IL-10 and mediated suppression of the type-1 inflammatory immune response [83]. Probably, the effect of these

Tregs goes beyond the sole local suppressive activity and the delay of the onset of immune response. The IL-10, TGF-β and CTLA-4 suppressive environment probably yields "special" conditions that favor the expansion of antigen-specific Tregs, as seen in some trypanosome (*T. congolense* and *T. cruzi*) mouse models [83, 88]. In addition, *T. cruzi* trypomastigotes seem to be responsible for the generation of regulatory DC *in vitro;* in turn, these DCs induce Treg cells *in vitro* [87]. The mechanism may involve DC activation by the parasite, mediated by TLR4 and ERK, and is associated to the yielding of high IL-10 levels [89]. Nevertheless, the relevance of this phenomenon in Treg induction remains to be elucidated.

In malaria

Malaria is caused by infection with protozoan parasites of the genus *Plasmodium*. After infection, sporozoites move from the dermis to the liver, where they go through an asymptomatic stage of rapid division before the parasite re-enters the bloodstream. In blood, exponential expansion of parasite populations leads to febrile illness. Typically, acute infection is controlled and chronic infection is established at reasonably low parasite density. However, in some cases the disease is lethal [90].

Infection with malaria parasites induces an immune response, either an effector or a suppressive one. Treg cells induction during malaria infection could be either beneficial or detrimental to the progression of the disease. Controversial results on the immunomodulatory properties of parasite components are reported. There is also conflicting information about the role of Tregs in malaria infection. In mice models, Tregs depletion protects experimental subjects from death when infected with a lethal strain of *Plasmodium yoelii*. Additionally, Tregs depletion protects mice from *Plasmodium falciparum* in cerebral malaria (CM) [91-93]. In contrast, during CM associated with *Plasmodium berghei* or during infection with *Plasmodium chabaudi,* Tregs depletion does not alter the parasitemia in mice [94-96]. These contradictory effects of Tregs in *Plasmodium* infection could be related to kinetic differences in the modulation of pro- and anti-inflammatory responses. Moreover, particular parameters such as mouse strain, age, sex, early cytokine profile production, and natural susceptibility to infection may influence the resulting role of Tregs.

In spite of the contradictory evidence on the role of Treg cells on malaria infection, some results suggest the Treg cell induction by *Plasmodium*. In fact, several reports demonstrate the increase of Tregs with suppressive capacity: Human infections with *Plasmodium falciparum* and *P. vivax* induced a significant expansion of CD4+CD25+Foxp3+ Treg cells expressing the key immunomodulatory molecule CTLA-4 [97]. Also, a Treg increase in human cord blood was observed in newborns of women with malaria; these Tregs are able to suppress Th1-type recall responses [98]. In addition, an increased proportion of Tregs with suppressive functions has been observed in *P. yoelii* and in *P. berghei* infected mice [95, 99-101]. The effect of this Treg increase at the beginning or during malaria progression needs to be clarified.

Moreover, malaria infection seemingly drives Tregs generation/induction. Couper *et al.*, 2007, have compared the depletion and repopulation of spleen CD4+CD25+Foxp3+ Tregs in uninfected and malaria-infected mice. By adoptive transfer, they have shown that repopulation of the spleen by CD25(high)Foxp3+ cells results from the re-expression of CD25 on peripheral populations of CD25-Foxp3+. Repopulation of the spleen by CD25+Foxp3+ cells occurs extremely rapidly in malaria-infected mice when compared to non-infected mice. Thus, malaria infection may drive proliferation and CD25 expression in peripheral CD4+CD25-Foxp3+ cells and/or conversion of CD4+CD25-Foxp3- cells [102].

On the other hand, T cell induction during malaria may be cytokine-dependent (IL-10 and TGF-β). A correlation has been observed between Tregs and elevated IL-10 levels in susceptible mice, but not in resistant mouse strains [101]. *P. yoelii* triggers Treg cell induction, probably mediated by TGF-β activation. In fact, thrombospondin-like molecules and metalloproteases present in *P. yoelii* extracts are able to activate latent TGF-β to its bioactive form. During the priming of malaria-specific T cells, TGF-β might up-regulate Foxp3, leading to the development of regulatory T cells [103].

In humans, a *P. falciparum*-mediated conversion of CD4(+)CD25(-) T cells into CD25+Foxp3+CD4+ T cells has been described. Induction of Foxp3 cells *in vitro* required TGF-β1 and IL-10, and these cells show the typical suppressor phenotype (CTLA-4(+), CD127(low), CD39(+), ICOS(+), TNFRII(+)) [104]. In accordance, an increase in systemic IL-10 and TGF-β that correlates with up-regulation of CD4+CD25+Foxp3+ has been observed in humans infected with *P. falciparum and P. vivax* [105, 106].

The induction of Treg cells during malaria may imply dendritic cells. During *Plasmodium yoelii* infection, regulatory $CD11c^{low}CD45RB^{high}$ DCs become prevalent in the spleen, overtaking the conventional $CD11c^{high}$ DCs. Additionally, regulatory $CD11c^{low}CD45RB^{high}$ DCs induce IL-10-expressing CD4 T cell [107]. *In vivo* experiments demonstrated

that during acute infection of *P. yoelii,* DCs migrate to the spleen and secrete TGF-β, prostaglandin E2 and IL-10. These DCs inhibit *Plasmodium* response, probably by generation of new Treg cells [108]. On the other hand, the pigment hemozoin (a metabolite from the parasite's intraerythrocytic stage) and *P. falciparum* merozoites promoted the generation of immunosuppressive dendritic cells. The maturation of human monocyte-derived dendritic cells (DCs) *in vitro* with hemozoin and merozoites prevented IL-12 production and augmented the release of IL-10 and TNFα. Additionally, merozoites prevented soluble CD40 ligand-induced phenotypic maturation of DCs [109, 110]. This observation suggests that hemozoin and merozoites induce a suppressive DC phenotype, which may play a role in favor of Treg cell induction. However, the immunosuppressive properties of hemozoin and merozoites remain uncertain. There are reports concerning the immunosuppressive properties of hemozoin and merozoites by inhibiting dendritic cell activity (hemozoin reviewed in [111]) [110]. In contrast, other authors suggest DC activation by these parasitic components [112-114]. Careful investigations are required to identify the molecule(s) responsible of modulating the DC function during malaria and their role in Treg induction.

On the other hand, it has been described that Treg cell induction can be a result of indirect interactions during malaria infection. Scholzen *et al.* reported an induction of human CD25+Foxp3+ CD4 T cells independent of MHCII interaction. Co-culture of the trophozoite-stage malaria-infected red blood cells with PBMC induces an enhancement of Treg levels. This induction seems not to be necessarily mediated by direct TCR stimulation, but could be mediated by soluble factors, including IL-2, IL-10, and TGF-β [104]. Also, Hisaeda observed that *P. yoelii* induces Tregs independently of MHCII interaction. Malaria parasites interact with TLR9 in DCs to induce Tregs during infection [115]. This author and some others have proposed that merozoites/protein-DNA complexes or genetic material trapped in hemozoin crystals are ligands for TLR9 [112-115]. Thus, TLR9 signaling might be a key factor for Treg induction [115] (Table 4). In contrast, other authors have observed that TLR9-deficient mice are able to trigger immune responses, control the infection and clear the parasite [113, 116]. Although the latter studies did not report Treg levels, this finding indicate the need to further study the role of TLR9 during this parasitosis and its effect on Treg induction.

Table 4. Regulatory T cells (Tregs) in malaria

Parasites	Host	Molecule involved	Cells involved	Role of Tregs in pathogenesis	Ref #
P. yoelii	Mouse	IL-10, TGF-β	‡Tregs, *Tr1	Contribute to immune suppression, impede parasite clearance and promotes mice death	[91, 146, 147]
P. yoelii	Mouse	IL-10	Tregs	Suppress Th1 response early in the infection	[101, 147, 148]
P. falciparum	Human	TGF-β,	Tregs	Increase *in vivo* parasite growth and disease severity	[105, 149]
P. falciparum	Human	IL-10, CTLA-4	Tregs	Suppress Th1 response and favor placental infection	[150, 151]
P. falciparum	Mouse	IL-10	Tregs	Modulate the pro- and anti-inflammatory responses affecting pathogenesis	[92]
P. berghei	Mouse		∞nTregs	Suppress the Th1 response	[93, 100]
P. berghei	Mouse		Tregs	No effect on cerebral malaria	[99]
P. chabaudi	Mouse		nTregs	Control the secretion of pro-inflammatory cytokines	[95]
P. vivax	Human	IL-10, TGF-β, GITR, CTLA-4	Tregs	Increase parasite burden	[106]
P. falciparum	Human	IL-10, TGF-β	Tregs	No effect on acute malarial inflammation Facilitate parasite clearance without causing immunopathology	[152]
P. yoelii	Mouse	TLR9	Tregs	Prevent anti-parasite immunity	[115]

*Tr1: IL-10 producing cells, ‡Tregs: conventional Foxp3+ cells, ∞nTregs: Natural Tregs

In nematode infections

The filarial worm *Onchocerca volvulus* has been shown that induces immunosuppressive environment by cytokines such as IL-10 and TGF-β [117, 118]. In fact, infected patients has Tregs expressing CTLA-4 in onchocercomas lesion; and this cells are associated to immunosuppression during chronic infection [119]. Concordantly, hyper-reactive patients show reduced TGF-β expression [120] indicating that this cytokine may participate in down-regulating the immune response.

Other human and animal filarial diseases (*Wuchereria bancrofti*, *Brugia malayi, Mansonella perstans* and

Litomosoides sigmodontis) promote Treg recruitment and increase Treg levels at the infection site (Table 5) [121-123]. In *Brugia malayi* infection (larvae and adult stages) there is immunomodulation associated to Treg expansion and to an increase in suppressive molecules such as TGF-β, CTLA-4, PD-1, ICOS, IDO [124, 125]. During *L. sigmodontis* infection, Tregs are increased both in acute and chronic infections [121, 126]. Taylor *et al.* observed that Treg depletion during *L. sigmodontis* infection did not affect larval establishment, but reduced the number of adult parasites [121]. Also, in *W. bancrofti and M. perstans* infection, Treg expansion has been observed, associated to an IL-10-dominated regulatory environment [122]. A variety of events may contribute to activation or control of the immune response. In fact, not only parasite components may control the immune response, but also other factors affecting the parasite itself. As an example, the endosymbiont *Wolbachia* (a bacterium living in filarial nematodes) is essential for the worm fertility and survival, and modulates the immune response in patients harboring *W. bancrofti*. In fact, a recombinant *Wolbachia* surface protein (WSP) induces suppressive T cell response in *Wuchereria bancrofti* infections. WSP components induce the expression of CTLA-4, IL-10, and TGF-β in PBMCs from filariasis patients [127]. This suggests that WSP may contribute to the suppression of the immune response in filariasis patients, probably by Treg induction.

Heligmosomoides polygyrus, a gastrointestinal nematode, induces *de novo* Foxp3 expression in T cells and increases IL-10 and TGF-β levels; these induced Tregs express CD103 and CTLA-4 [128]. Additionally, induced Tregs have suppressive activity *in vitro* [128, 129]. Rausch *et al.* have examined the role of effector and regulatory T cells during *H. polygyrus* infection by adoptive transfer. Their results show that while conventional T effector cells conferred protection and led to a significant decrease in worm burdens, Tregs had no effect on worm burdens in the host. However, not long after infection, the number of Foxp3+ Treg cells temporarily increased in the inflamed tissue. On the other hand, the transfer of a heterogeneous T-cell population containing Treg and T effector cells yields a significant decrease in worm number [129]. This study points out the importance of a balance between effector and regulatory immune response to determinate the course of infection.

Table 5. Regulatory T cells (Tregs) in nematode infections

Parasites	Host	Molecule involved	Cells involved	Role of Tregs in pathogenesis	Ref #
O. volvulus	Human	IL-10, TGF-β, CTLA-4	*Tr1	Tregs in onchocercomas are associated with immunosuppression during chronic infection.	[119]
L. sigmodontis	Mouse	IL-10, CTLA-4, GITR	‡Tregs	No effect on larva establishment but favors chronicity. Inhibit protective immunity	[121, 126]
L. sigmodontis	Mouse	TGF-β	Tregs	Induce unspecific suppression	[153]
W. bancrofti and M. perstans	Human	IL-10	∞nTregs, Tr1	Induce a regulatory environment	[122]
W. bancrofti	Human	§WSP, IL-10, TGF-β, CTLA-4	T cell	Induce hypo-responsiveness in antigen-specific T cells	[127]
B. malayi	Human	TGF-β, CTLA-4, PD-1, ICOS, IDO	Tregs	Impairment of specific Th1 and Th2 immune response	[124]
H. polygyrus	Mouse	IL-10, TGF-β, CTLA-4	Tregs	No effect on worm burdens	[129]
H. polygyrus	Mouse	IL-10, TGF-β	Tregs	*In vitro* suppression of effector response	[128]
H. polygyrus	Mouse	**HES	DC	Impairment of dendritic cell function and modulation of immune responses	[131]
H. polygyrus	Mouse	HES	Treg	Promote suppressive immune response	[132]

*Tr1: IL-10 producing cells, ‡Tregs: conventional Foxp3+ cells, ∞nTregs: Natural Tregs, §WSP Wolbachia surface protein, **HES: Parasite excretory-secretory products.

The mechanisms of Treg induction in nematode infections are not known. However, a mechanism for Treg induction by *Heligmosomoides* has been described, and it has been proposed to be common among nematodes. On one side, the secreted and constitutive parasite components act on DCs promoting Treg generation. On the other side, secreted components act directly on T lymphocytes to turn them into Tregs [130]. In fact, *H. polygyrus* produces *in vitro* an excretory-secretory antigen termed HES. It has been described that HES and an adult worm homogenate (AWH) inhibit DC activation, cytokine (IL-12p70, IL-6, TNF-α, and IL-10) and chemokine (MCP-1, and RANTES) production, as well as co-stimulatory molecule expression (CD40, CD86 and MHC class II). Also, in DC HES induce the generation of CD4+CD25+ IL-10-producing T cells able to inhibit

effector T cell proliferation and IFN-γ production [131].

With regard to the effect of HES in T cells, Grainger *et al*. have described that HES is able to ligate directly to TGF-β receptor in T cells and promote a signaling pathway (Smad2/3 phosphorilation). Thus, HES triggers a TGF-β-like activity that mimics TGF-β functions. More interestingly, HES induces *de novo* Foxp3 expression in naïve T cells depending on signaling through the TGF-β receptor. To confirm the role of HES in Treg induction, the authors show that Foxp3 Treg cell conversion is enhanced *in vivo* by *H. polygyrus* infection [132]. The fact that nematodes secrete a TGF-β-like molecule suggests that parasites have developed strategies to circumvent the host's immune response. These parasites have been learned during millions of years of co-evolution with their hosts to identify molecules that promote Treg induction.

Conclusions

Parasites use all the mechanisms herein described to induce Treg cells during their establishment and to promote their survival in the host. However, the structure and function of the parasite molecules involved in Treg induction is just partially known. Many parasitic components could act as "cytokine-like" or directly stimulate immune receptors; it is also possible that parasite molecules act directly as Treg cell inducers. Some cases have been described in which a parasite molecule can use TLR4, TLR2 and TLR9 as intermediaries to induce Treg cells [89, 115, 133]. In addition, parasites drive the cytokine balance during infection to yield an environment that promotes Tregs generation. The knowledge on the immune components and parasite molecules involved in controlling the immune response would be useful for a better understanding of the molecular mechanisms of Treg induction. Finally, the use of parasite molecules to induce Tregs *in vivo* may provide a new alternative to control inflammation in other diseases.

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Abbreviations

Tregs: regulatory T cells; Tr1: T regulatory 1 cells; Th3: T helper 3 cells; TCR: T cell receptor; PDL1: programmed death ligand 1; TGF-β: transforming growth factor-β; DC: dendritic cells; APCs: antigen-presenting cells; SOCS: suppressors of cytokine signaling; STAT: signal transducer and activator of transcription; GCs: glucocorticoids; GILZ: glucocorticoid-induced leucine zipper; pDCs: plasmacytoid DCs; IDO: indoleamine 2,3-dioxygenase; ICOS: inducible T-cell co-stimulator.

Conflict of Interests

The authors have declared that no conflict of interest exists. The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflicts with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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