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The Role of Regulatory $CD4^+CD25^+$ T Cell Subset in Host Homeostasis during Protozoan Infection: An Overview

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***Abstract*— Human diseases caused by protozoan parasites are renowned for their high rates of morbidity and mortality worldwide. Some examples include African trypanosomiasis or sleeping sickness, American trypanosomiasis or Chagas disease, leishmaniases, malaria and babesiosis. These infections tend to follow a chronic rather than an acute course with lifelong persistence of parasites. Regulatory T cells (Treg), in particular the $CD4^+CD25^+$ cell subset, appear to control the immune competence of host response triggered by the presence of parasites, promoting homeostasis and protecting the host from collateral tissue damage whilst allowing parasite persistence. To date, there is still considerable controversy on the characteristics and function of these cells when induced during different protozoan infections, evidencing the need of further research. Therefore, this review aims to provide a comprehensive overview about Treg cells development, phenotype determination and general functions. The above pathologies were used as selected examples to discuss the role of Treg cells during protozoan infections. Understanding of the mechanisms that contribute towards homeostasis and the survival of the host, and simultaneously allow the persistence of the pathogen, may yield important insights for new strategies of prophylaxis and therapy.**

***Keywords* — Protozoan infections; Regulatory T cells; $CD4^+CD25^+FoxP3^+$ T cell subset; *Trypanosoma* spp.; *Leishmania* spp.; *Plasmodium* spp.; *Babesia* spp.**

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I. Introduction

PROTOZOAN parasites are the causative agents of many insect-borne infections that continue to represent major threats to human health worldwide [1]. In developing countries, these diseases constitute an important cause of morbidity and mortality [2, 3], mainly due to the limited availability of therapies and lack of affordable and effective vaccination strategies [4, 5]. Some examples include African trypanosomiasis or sleeping sickness, American trypanosomiasis or Chagas disease, leishmaniasis, malaria and babesiosis, which are caused by *Trypanosoma brucei*, *Trypanosoma cruzi*, *Leishmania* spp., *Plasmodium* spp., and *Babesia* spp., respectively.

In immunocompetent individuals, despite the existence of a strong anti-protozoan response, the immune system seems to allow the persistence of a small number of parasites, with parasite load persisting for a long time at a tolerable level. Thus, the infections tend to follow a chronic rather than an acute course [6], as sterile immunity is difficult to achieve, which could be beneficial for the host in endemic areas, leading to the establishment of protective cell memory [7]. In addition, since these parasites are able to establish a persistent infection, situations that disturb the immune system, such as malnutrition, advanced age or co-infections can lead to the reactivation of these infections. More recently, an emergent problem has arisen in humans regarding acquired immunodeficiency, caused for example by Human Immunodeficiency Virus (HIV) or by specific immune suppressor treatments, responsible for an increase of immune compromised individuals. In these individuals the normally asymptomatic or latent protozoan infections can be triggered and cause significant clinical disease (reviewed by Mendez et al. [8]).

Over recent years, the concept of a self-regulating immune system has been researched extensively. The potency of the host immune response triggered by the presence of parasites has to be strongly counterbalanced by regulatory responses that control the extent of auto-reactive responses and promote homeostasis during infection. It is therefore important to achieve effective parasite control whilst protecting the host from tissue damage, preventing clearance of infection [6, 9, 10]. If uncontrolled by the regulatory mechanisms, the induction of a strong immune response can cause immunopathology and even lethality [9]. Amongst others, one such mechanism involves the generation of immunosuppressive regulatory T cells (Treg) [11]. Under

normal conditions, Treg cells can regulate the innate and adaptive immune responses and control the excessive and misdirected response mediated by effector T cells [12, 13].

This study aims to review the published information about the induction and the role of Treg cells, in particular the $CD4^+CD25^+$ Treg cell subset, in maintaining homeostasis during the immune response triggered by the presence of protozoan parasites responsible for trypanosomiasis, leishmaniasis, malaria and lastly babesiosis. In order to understand how the regulatory mechanisms exert their action, a brief overview describing the development of Treg cells, phenotype determination and general functions is provided. Finally, an understanding of the mechanisms by which regulation of the immune system ensures homeostasis and avoids severe immunopathology of the host by suppressing immune response, and simultaneously allows persistence of the pathogen, may yield important evidences to incorporate into the design and development of novel strategies for prophylaxis and therapeutics.

II. The Role of Regulatory $CD4^+CD25^+$ T Cells: An Overview

A. Regulation of the immune system: Regulatory T cells

A large and ever increasing body of data is accumulating on a specialized subset of Treg cells, the $CD4^+CD25^+$ T cells, also known as natural or endogenous regulatory T cells (nTreg). In healthy humans, these cells comprise 2-4% of peripheral $CD4^+$ T cells, whereas in mice represent 5 to 10% [14]. This subset of cells is able to prevent autoimmunity through inhibition of the activation and expansion of autoreactive T cells and actively maintain peripheral tolerance [12, 15, 16]. Treg cells are able to recognize self-antigens in autoimmunity, as well as exogenous antigens in hypersensitivity and infectious diseases [17, 18].

B. Development of regulatory T cells

Uncommitted thymocytes differentiate into either a $CD4^+$ or a $CD8^+$ thymocyte. Within the thymus, nTreg cells develop from naïve $CD4^+$ thymocytes following exposure to antigen and self-stimulation with cytokines [19]. This yields a fully functional mature subset of T cells which migrate to the periphery and are immediately able to suppress auto-reactive conventional T cells. nTreg cells exhibit a characteristic immune phenotype which include the expression of the transcription factor forkhead box P3 (FoxP3) [20] and of the surface markers cytotoxic T-lymphocyte antigen 4 (CTLA-4), CD28, glucocorticoid-induced TNF receptor-related protein (GITR) and the interleukin (IL)-2 receptor α (CD25) [21]. In addition to thymically derived Treg cells, naïve lymphocytes (both $CD4^+$ and $CD8^+$) move to peripheral sites and undergo maturation to produce further distinct subsets. Naïve $CD8^+$ T cells develop into a minor Treg population, which may or may not express FoxP3, following exposure to foreign or self-antigen [22]. By contrast, the naïve $CD4^+$ cells that have migrated are induced through exposure to a low dose of antigen alongside stimulation with the cytokines IL-2 and transforming growth factor (TGF)-β [23, 24]. These cells commonly referred to as "induced" Treg cells (iTreg) express FoxP3 and maintain peripheral tolerance [24]. Mucosa-associated lymphoid tissues (MALT) are further sites where the development and expansion of distinct subsets of Treg cells can be driven by cytokines. MALT-resident antigen presenting cells (APC) secrete IL-10, TGF-β or a "cocktail" of IL-2/TGF-β/retinoic acid, which, respectively, leads to the differentiation of naïve $CD4^+$ T cells into regulatory T cells of type 1 (TR1) [25], T helper 3 cells (Th3) [26] and iTreg cells [27]. When exposed to the altered microenvironment created under certain pathological conditions, Treg cells can lose FoxP3 expression and consequently, their ability to suppress other cells, behaving like conventional effector T cells. All these potential outcomes generate an enormous plasticity within the peripheral pool of T cells [28]. In addition, the *in vitro* stimulation of Treg cells with IL-6 causes the loss of FoxP3 and differentiation into T helper 17 cells (Th17) that secrete IL-17 [29].

C. Function of Regulatory T cells

The major function of Treg cells is to maintain immune cell homeostasis. This is achieved via four alternative mechanisms that negatively regulate conventional T cells and dendritic cells (DC), although other major groups of immune cells such as natural killer (NK) cells can also be inhibited. One such mechanism involves the use of suppressive cytokines or other molecules, which are either secreted or expressed on the surface of Treg cells. Contact-dependent inhibition of $CD4^+$ or $CD8^+$ effector T cells is achieved through exposure to TGF-β attached to the Treg cell membrane [1]. Treg cells can also secrete IL-10, IL-35 or TGF-β, which induce cell cycle arrest in effector T cells located nearby [2]. The secretion of IL-10 can also block co-stimulation of DC. Furthermore, galectin-1 can be released by Treg cells to induce apoptosis in adjacent T cells expressing the CD45 and CD43 glycoprotein receptors.

A second mechanism adopted by $CD8^+$ Treg cells also induces apoptosis in either T cells or DC. The secretion of perforin molecules creates pores in the membranes of effector T cells or DCs and granzymes (A or B) giving origin to the cascade of caspases, ending in programmed cell death (reviewed by Sakaguchi et al. [2]).

A third mechanism of Treg-mediated suppression involves disrupting the metabolic function of target cells. There are three well-defined ways by which this is achieved. Firstly, Treg cells are capable of depriving conventional T cells of a source of IL-2, attenuating their proliferation, [32] leading to apoptosis. Secondly, Treg cells can initiate indirect apoptosis of T cells via a DC intermediate. In this situation, CTLA-4 expressed on the surface of Treg cells binds to either CD80 or CD86 molecules found on DC, upregulating indoleamine 2,3-dioxygenase production in DC [3]. This enzyme catabolises tryptophan and the simultaneous depletion of this essential amino acid starve nearby T cells, directing them to apoptose. Finally, the CD39 and CD73 glycoproteins on the surface of Treg cells synthesize adenosine from adenosine triphosphate in the extracellular space [4]. Both DC and conventional activated T helper cells express the adenosine receptor A2AR, and following binding of the nucleoside, are

inhibited. In the case of DCs, their maturation and ability to stimulate T cells is suppressed. Helper T cells are suppressed by adenosine through its effect on their production of cytokines. A significant reduction in the secretion of IL-2, IL-4, tumor necrosis factor (TNF)-α and interferon gamma (IFN)-γ is observed due to decreased transcript stability in these cells [4].

A fourth mechanism of $FoxP3^+$Treg cells mediated suppression consists of alternative ways to target DC, of which three different contact-dependent mechanisms have been defined. Firstly, $FoxP3^+$ Treg cells use their T-cell receptors to engage class II molecules of major histocompatibility complex (MHCII) on the surface of DC. Coupled with the binding of Neuropilin-1 on the Treg cell surface to its similar receptor found in DC, prolongs their interaction at the immunological synapse, outcompeting nearby T helper cells and causing the suppression of APC [5]. A second way implies targeting of DC and inhibition of cell maturation. Here, the lymphocyte activation gene 3 transmembrane protein expressed by $FoxP3^+$ Treg cells binds to MHCII on the surface of DC, inhibiting their activation [6]. The third alternative DC-targeting mechanism employed by iTreg cells uses the CTLA-4 receptor present in membrane. This constitutively expressed protein binds to CD80/CD86 molecules on the DC surface which leads to their internalization in a process termed trans-endocytosis [7] inhibiting DC function.

D. Regulatory T cells preventing autoimmunity

Treg cells have been identified as the key players in a number of important immune responses *in vivo*. One such example is the prevention of autoimmunity whereby Treg cells suppress the activation of the effector T cells. In mice depleted of Treg cells, autoimmunity prevails, however, the reconstitution of this $CD4^+CD25^+$ T cell subset prevents autoimmunity [8]. Nonetheless, more recent evidence has indicated that effector T cells are able to resist this suppression. For example, in patients with rheumatoid arthritis [9] and multiple sclerosis [10], Treg-mediated suppression is lost and peripheral blood Treg cell counts are similar between patients and healthy controls. A recent study has deciphered the underlying mechanism responsible for this absence of regulation. In patients with juvenile idiopathic arthritis, resistance to Treg suppression is mediated by hyperactivation of protein kinase B in effector T cells at the site of inflammation [11]. This is a direct consequence of these cells being stimulated by IL-6 and TNF-α, though other cytokines and CD28 signaling may also contribute [11].

E. Regulatory T cells promoting chronic infection

Another important manifestation influenced by $CD4^+CD25^+FoxP3^+$ Treg cells is the establishment of chronic infection. A broad spectrum of pathogens, including bacteria (*Helicobacter pylori*), fungi (*Candida albicans*), protozoan (*Leishmania major*) and virus (hepatitis C virus) has been shown to become manifested as persistent human infections because the action of Treg cells [12].

Most protozoan infections follow a chronic rather than an acute course, with the parasite persisting for a long time [13, 14]. To achieve effective parasite control, whilst protecting the host from tissue damage, a fine balance needs to be achieved by Treg cells to limit the potency of the host immune response. An overabundance of Treg cells or their overactivation may cause immunopathology and lethality and, also allow pathogens to become transmitted more easily [15]. Somewhere between these two extremes exists a balance between the immunosuppressive activity of Treg cells and effector T cells. Although the host may benefit from milder pathology and be protected from disease, this can be where infections persist and become established as chronic diseases [12].

Despite the complex interactions between parasites and their hosts, considerable progress has been made to understand the involvement of Treg cells during many protozoan infections. Though their role is not very well defined, some illustrative examples of protozoan infections that involve Treg cells in their pathogenesis are reviewed.

III. The Role of Regulatory $CD4^+CD25^+$ T Cells Subset in Host Homeostasis During Protozoan Infection: Leishmaniasis, Trypanosomiasis, Malaria and Babesiosis

A. Leishmaniasis

Leishmaniasis, caused by *Leishmania* spp., is a serious public health issue with a worldwide distribution [16]. *Leishmania* includes a broad genus of obligatory intracellular flagellate protozoa that infect mononuclear phagocytes [17].

Leishmania uses a very distinct strategy to preserve their life cycle based on just two morphological forms: the amastigote, a non-flagellated form that resides intracellularly within the mononuclear cells of their mammalian hosts, such as humans, dogs and rodents, among others; and the promastigote, an extracellular flagellate form that develops in the intestinal tract of sandfly. The vector, a female sandfly of the genus *Phlebotomus* and *Lutzomyia* ingests amastigote forms while feeding on the mammal host and, within the midgut, the amastigote forms develop into infective procyclic promastigote forms [18]. In a subsequent blood meal, the female sandfly deposits the promastigote forms into a mammalian host [19]. Shortly after inoculation, these promastigote forms are phagocyted by dermal macrophages and/or DC at the site of the bite and, inside phagolysosomes, they transform into amastigotes and replicate [17, 19]. The excess of parasite load inside the cells due to parasite replication within the phagolysosome leads to cell lysis and the free amastigotes will then infect new phagocytes. The life cycle is completed when the female sandflies ingest infected phagocytes [18].

Leishmania spp. are responsible for a wide variety of disease presentations, namely the polymorphic cutaneous forms, the rare mucocutaneous and the fatal disseminated visceral form [17]. Clinical symptoms are caused by parasite dispersion within macrophages by nasopharyngeal mucosa and by organs of the mononuclear phagocytic system (reviewed by Campanelli et al. [20]).

Cutaneous leishmaniasis can be caused by *L. major*, *L. tropica* and *L. aethiopica* in Africa, Middle East and parts of Asia [17], and by several *Leishmania* species of the braziliensis and mexicana complexes in central and South

America. In most vertebrate hosts, the infection is characterized by a period of latency, followed by lesion development that may not ulcerate [15].

During infections caused by *Leishmania* spp., in particular cutaneous leishmaniasis, regulation of the immune response is important for two reasons: firstly, it allows the persistence of the parasite within the host cells after clinical resolution; and secondly, contributes to the establishment of the host cell memory [21], and eventually protection against re-infection. After successful chemotherapy or self-cure, low numbers of viable parasites will persist within lymphoid tissue and at the skin lesions, giving rise to latent infections [22, 23]. Macrophages and DCs were found to harbor persistent parasites within the lymph nodes [24, 25]. This latent infection can be reactivated as a result of immunosuppression, environmental factors or with advanced age (reviewed by Mendez et al. [23]) and give rise to visceral leishmaniasis and mucosal leishmaniasis (reviewed by Belkaid et al. [22]). The equilibrium established during chronic infection might reflect both parasite and host survival strategies. From the host point of view, the inability to achieve an absolute cure is beneficial, especially if they reside in endemic areas, where an efficient memory response is important [21, 26].

Different studies support the fact that Treg cells, in particular the $CD4^+CD25^+$ subset, have a fundamental role controlling the outcome of *Leishmania* infection, among various immune mechanisms. A study determined that $CD4^+CD25^+$ Treg cells are essential for the development and maintenance of a persistent cutaneous infection with *L. major* in resistant C57BL/6 mice. Treg cells rapidly accumulated at primary sites of infection, suppressing the capacity of the immune response to completely eliminate the parasite [26]. In humans with cutaneous leishmaniasis, $CD4^+CD25^+$ Treg cells were found in chronic skin lesions caused by *L. braziliensis* [20].

Treg cells can also control the intensity of the memory response and the balance between these cell subset and effector T cells seems to affect the course of the disease. Mendez et al. [23] found that during memory responses, the number of $CD4^+CD25^+$ Treg cells increased at sites where the infection was reactivated. Similarly, the presence of Treg cells during human *L. major* infection was confirmed to be higher in chronic lesions in comparison to effector T cells, which in turn were more frequent in active lesions [27]. Overall, results show that the balance between Treg and effector T cells determines the outcome of infection. The balance tendency towards Treg cells in chronic infections and their presence at the time of memory response might be the cause of long-lasting disease and a decisive factor governing the efficiency of effector immune responses.

$CD4^+CD25^+$ Treg cells produce the immunosuppressive cytokine IL-10 that is now known to play a role in parasite persistence. Belkaid et al. [22] investigated the factors that control *L. major* persistence and reactivation after clinical cure. Comparing acute and chronic infections in C57BL/6 and C57BL/10 wild-type mice and in IL-10 knockout mice, they have found that IL-10 is required for *L. major* persistence. During the chronic stage, IL-10 deficient mice and wild-type mice treated with anti-IL-10 antibody achieved sterile immunity, with complete clearance of parasites from the skin. Kane and Mosser [28] confirmed the role of IL-10 in cutaneous leishmaniasis. After *L. major* infection, it was shown that genetically susceptible BALB/c mice lacking IL-10 were able to control disease progression, developing relatively small lesions; however, BALB/c wild-type mice developed progressive non-healing lesions with numerous parasites [28]. More recently, the production of IL-10 by $CD4^+CD25^+$ Treg cells was confirmed in C57BL/6 mice infected with a strain of *L. major* responsible for non-healing dermal lesions [29]. The results support the fact that IL-10 exerts a potent inhibitory effect on macrophage activation, especially at the level of cytokine production [30] and leishmanicide capability [31, 32].

Treg cells can downregulate both protective T helper (Th) 1 and pathogenic Th2 responses and the balance of Th1 and Th2 cell functions is critical for the outcome of cutaneous leishmaniasis. Belkaid et al. [26] have shown that in genetically resistant mice, $CD4^+CD25^+$ Treg cells suppress Th1 cells, preventing the parasites from being completely eliminated whilst in susceptible mice, Treg cells prevent the early appearance of lesions and lead to a better control of parasites in the long term. $CD4^+CD25^+$ Treg cells can also suppress the differentiation of Th2 cells and prevent the development of progressive lesions caused by *L. major* in susceptible mice [33]. In addition, after *L. major* infection, BALB/c mice depleted of $CD4^+CD25^+$ Treg cells, in comparison with wild-type BALB/c mice, develop more severe lesions with higher numbers of parasites, probably because $CD4^+CD25^+$ Treg cells downregulate IL-4 secretion by $CD4^+$ T cells, thus affecting Th2 cell maturation [34].

Visceral leishmaniasis, commonly known as Kala-azar, is caused by *L. donovani* in South Asia and Africa and by *L. infantum* in the Mediterranean region, the Middle East, Latin America and some areas of Asia [17]. Parasite replication within macrophages with further spreading to other locations of the mononuclear phagocytic system is responsible for disease pathogenesis [16].

During murine leishmaniasis, $CD4^+CD25^+$ Treg cells also seem to play a role during the course of *L. infantum* infection, promoting parasite persistence and the establishment of chronic infection. After experimental infection of susceptible BALB/c mice with *L. infantum*, it was observed that the $CD4^+CD25^+$ Treg cells rapidly expand in infected lymph nodes and spleen cells along with an increased parasite load. In addition to which, several characteristic Treg cell surface markers (FoxP3, CD25, GITR, CD103) were concurrently transcribed at 7 days after infection, underlining the involvement of these cells during this infection. At this specific time point, a substantial decrease in IL-4 secretion from the $CD4^+CD25^-$ effector T cell subset harvested from the lymph nodes and splenocytes of infected mice was observed. This suggests that Treg cells undergoing expansion during this stage of the infection were suppressing the Th2 effector cell population. A statistically significant increase in IL-10 production by these same $CD4^+CD25^-$ T cells was also seen at 7 days after infection which may indicate the TR1 regulatory subset contribute, in part, to the suppression of effector T cells [16].

B. *Trypanosomiasis*

Trypanosoma brucei gambiense and *T. brucei rhodesiense* are flagellate parasites responsible for the African trypanosomiasis in humans, also known as the sleeping sickness [35]. *T. brucei* gambiense is frequently found in West and Central Africa, being responsible for a chronic form of the disease that can develop over months and years. *T. brucei rhodesiense*, a less common species, is often found in East Africa and is associated with severe acute forms of the disease. African trypanosomiasis caused by *T. b. rhodesiense* is a zoonotic disease, in which asymptomatic wild and domestic animals act as reservoirs [36].

African trypanosomes go through several morphologically distinct phases during their life cycle. The infection is initiated when a haematophagous arthropod, the tsetse fly (genus *Glossina*), feeds on a vertebrate host. During the blood meal, the tsetse fly deposits metacyclic trypomastigotes in the dermal connective tissue of the host. The parasites rapidly reach the lymphatic and blood circulation, replicate and develop into trypomastigotes spreading to other locations [37]. The tsetse fly becomes infected during feeding from an infected host. Then the trypomastigotes replicate in the midgut and develop into procyclic trypomastigotes. They subsequently develop into migrating epimastigotes that attach to the salivary glands, differentiating into metacyclic trypomastigotes to continue the parasite life cycle in a subsequent meal [36].

African trypanosomes are very unusual amongst protozoan parasites in that they only have parasitic extracellular stages in the vertebrate host, persisting for extended periods of time in the blood [37]. This characteristic makes them vulnerable to antibody-mediated immune destruction and around 99% of the circulating parasites are cleared from the blood by macrophages [14, 35].

Similarly to what has been described for *Leishmania* infections, some groups have shown that Treg cells are present and might play a pivotal role controlling the outcome of African trypanosomiasis and the persistence of the parasite. In trypanosomiasis, the persistent infections are accompanied by a profound immunosuppression (reviewed by Adalid-Peralta et al. [38]).

During African trypanosomiasis, it has been reported that the expansion of Treg cells is responsible for parasite tolerance from the moment of establishment of infection and continuing during chronic stage [38].

Guilliams et al. [39] showed that Treg cells influence the outcome of the disease in trypanotolerant C57BL/6 mice. C57BL/6 mice are considered tolerant to the disease when infected with *T. congolense*, controlling the first peak of parasitemia and developing a chronic and systemic infection. Their results have shown an expansion of $CD4^+CD25^+FoxP3^+$ Treg cells within the $CD4^+$ T cell population, both in the liver and spleen of infected mice. The expansion was verified after the first peak of parasitemia (acute phase) and during the chronic phase. It was also demonstrated that IL-10 produced by $CD4^+CD25^+$ Treg cells contribute to the suppression of IFN-γ generated by $CD4^+$ and $CD8^+$ T effector cells, as well downregulating the activation of macrophages. When Treg cells were depleted, using an anti-CD25 monoclonal antibody (IL-2Rα), the trypanotolerant C57BL/6 resistant mice decreased their survival rate with augmented liver necrosis and consequent loss in parasite clearance capacity [39]. These results show that Treg cells play a fundamental role in the downregulation of Th1 immune response during the chronic stage of infection, preventing injury to the liver, maintaining its parasite clearance function, and contributing to the development of the trypanotolerant phenotype in *T. congolense*-infected mice [39]. Finally, Treg cells may not only influence the local suppressive activity and the delay of the immune response in *T. congolense* infections, but also the secretion of IL-10. In turn, it is possible that IL-10 generate a beneficial suppressive environment for the expansion of antigen-specific Treg cells.

More recently, the same group has shown that sustained inflammation responsible for tissue damage and reduced survival of *T. brucei*-infected C57BL/6 mice undergoing a pathogenic infection was associated with the absence of Treg cell expansion [40].

Nevertheless, the contribution of $CD4^+CD25^+$ Treg cells to the survival rate and parasite persistence during *Trypanosoma* spp. infections is still controversial. Studies have shown that $CD4^+CD25^{high}FoxP3^+$ Treg cells prevent an early protective response in *T. congolense*-infected susceptible BALB/c mice. After an optimal dose of anti-CD25 antibody, infected mice did not develop parasitaemia, eliminated all parasites and showed no signs of disease [41].

American trypanosomiasis or Chagas disease is caused by the obligate intracellular parasite *Trypanosoma cruzi* [36].

Insects of the family Reduviidae become infected with the trypomastigote form when take a blood meal from an infected mammalian host. Replicative non-infective epimastigotes develop into trypomastigotes in the insect midgut. During the bite, the insect normally urines and defecates leading to the release of infective metacyclic trypomastigote forms [18]. The parasite then has access to host tissues through a continuity lesion on the skin caused by the insect, or through intact mucosae or conjunctivae [42]. Humans can also be infected by the oral route with subsequent invasion of the stomach epithelium or through the transplacental route (reviewed by Walker et al. [18]). *T. cruzi* is internalized either by nucleated host cells or phagocytized by macrophages and dendritic cells, with further proliferation into non-flagellated amastigotes inside cells from the skeletal muscle or myocardium [42]. After replication, amastigote forms are triggered to differentiate into motile flagellated trypomastigotes [18]. The infected cells rupture releasing new trypomastigotes into the blood and lymph, with consequent invasion of new cells [42]. The life cycle is complete when trypomastigotes or amastigotes, also known to be infective, are ingested by the insect during a blood meal and become epimastigotes again [18, 42].

The acute phase, usually not fatal, is followed by the chronic and asymptomatic phase, during which parasite multiplication seems to be controlled by the host immune system [15, 43]. The parasite is not completely eliminated, being able to persist in low numbers lifelong [15, 36, 43]. Human hosts will then serve as reservoirs [15, 43].

Replication control and persistence of the parasite during the chronic phase is mainly due to T cell-mediated immunity

regulating the anti-*T. cruzi* immune response [15, 44].

During cruzi-trypanosomiasis, Treg cells are present but their role in the control of the immune system is still controversial. Contrary to African trypanosomes, *T. cruzi* is an obligate intracellular parasite [36]. Thus, the immune response induced by an extracellular or by an intracellular parasite will probably trigger different regulatory mechanisms. Vitelli-Avelar et al. [44] were the first to document a high frequency of $CD4^+CD25^{high}$ Treg cells in whole blood obtained from chronic (asymptomatic) *T. cruzi*-infected human patients. Later, these results were supported by the increased concentration of $CD4^+CD25^+FoxP3^+$ Treg cells found in chagasic patients along with increased production of IL-10 and CTLA-4 [45, 46]. Furthermore, $CD4^+CD25^+$ Treg cell subsets expressing characteristic Treg cell markers, such as $FoxP3^+$, GITR and CTLA-4 were found in the heart of *T. cruzi* infected mice associated with host resistance and control of parasite replication. When treated with anti-GITR antibody to block the suppressor activity of Treg cells, mice suffered from increased parasitism, myocarditis and mortality alongside with upregulated production of TNF-α, in comparison with controls [47]. Taken together, these results suggest that Treg cells might play a role in the immune response against *T. cruzi* infection reducing extensive effective immune response, ultimately controlling the infection.

C. Malaria

Plasmodium spp. are parasitic protozoan responsible for causing malaria in the tropical and sub-tropical regions worldwide [48]. Currently, five species of the genus *Plasmodium* are known to infect between 300 and 600 million humans every year, resulting in approximately 1 million deaths [49]. In developing countries such as sub-Saharan African countries, the majority of the infections are caused by *P. falciparum* responsible for cerebral malaria, the most malignant form of the disease, which in most cases leads to death due to severe anemia and neurological problems [21, 48]. Malaria caused by *P. vivax*, known as relapsing malaria, and *P. ovale*, together with *P. falciparum*, are the most widespread across continents. However, after a deep analysis Lysenko and Bejaev [50] report the presence of *P. ovale* mainly in the sub-Saharan Africa and the islands of the western pacific. Other publications also report its presence in New Guinea and the Philippines (as reviewed by Collins & Jeffery [51]). *P. malariae* and *P. knowlesi* have also been associated with cases of malaria in Southeast Asia [52]. In most cases and with the exception of a few regions, the acute form of the non-falciparum malaria is mild and can be controlled, persisting a chronic infection of low parasitemia [53].

The life cycle of *Plasmodium* spp. parasites is complex with different stages both in the anopheline vector and in the human host. In general, the infection starts when a female mosquito of the genus *Anopheles* feeds on a vertebrate host, inoculating sporozoites into the host bloodstream [18]. Sporozoites, the infectious form of the parasite, rapidly reach the liver, invade and multiply within the hepatocytes, originating the schizonts. Schizonts rupture releasing merozoites into the blood stream [48] that easily adhere to and infect red blood cells (RBC). Inside RBC the parasite expands exponentially, developing into the trophozoite stage [38], divide asexually into mature schizonts (erythrocytic schizogony), which in turn rupture the RBC and the merozoite form is released into the blood stream, consequently infecting other RBC, perpetuating the infection [18]. Some of the merozoites forms may develop into male and female gametocytes, the sexual erythrocytic stages. When ingested by a female mosquito, these forms fuse in the mosquito stomach to form motile zygotes that invade the midgut and develop into oocysts that after rupture release new sporozoites. The sporozoites migrate to the salivary glands and when the anopheline female takes up a blood meal on a vertebrate host, the parasite is transmitted continuing the life cycle [18, 48, 54].

Pathogenesis in malaria occurs after merozoites develop in RBC and the release of RBC cellular content activates monocytes and macrophages [52]. Splenic and circulant monocytes and DC activate $CD4^+$ Th1 effector cells [55, 56], which secrete pro-inflammatory cytokines, such as IFN-γ [57], TNF-α, IL-1β and IL-16 [58]. As with other protozoan parasites, host protection is rather ineffective, as *Plasmodium* parasites develop evasive mechanisms to hide from the immune system or subvert its mechanisms of action (reviewed by Coban et al. [59]), limiting adaptive immunity and memory acquisition. Furthermore, difficulties in understanding the disease are also related to external and extrinsic factors of the host itself which influence the level of pathogenesis, such as nutritional status [60], intestinal flora [57], age [55, 61, 62] and ethnicity.

The beginning of infection promotes the activation of the $CD4^+CD25^+FoxP3^+$ T cell subset [57, 63], inducing tolerance and promoting homeostasis [64] through the production of anti-inflammatory cytokines IL-10 and TGF-β. T lymphocyte receptors (TLR) on nTreg cells might also be stimulated by *Plasmodium* lipopolysacharide agonists, such as glycosylphosphatidylinositol (GPI) or hemozoin-bound DNA [65], and *P. falciparum* erythrocytic membrane protein 1 (PfEMP-1), presented by DC and macrophages [56]. Uncomplicated malaria by *P. falciparum* and *P. vivax* was also shown to induce changes in cell number and proportions of Treg cells and DC [66].

In human malaria, the suppression of host protection is probably mediated by IL-10 [60]. IL-10 origin is still unclear, but most likely secreted by different peripheral blood mononuclear cells (PBMC), such as monocytes [49, 60], macrophages [52], naïve and effector $CD4^+$ T and iTreg cell subsets [49]. Portugal et al. [67] also identified IL-10-producing $CD3^+CD4^+$FoxP3-PBMC induced by *P. falciparum* following fever in Malian children; whereas $CD4^+CD25^+FoxP3^+$ cells were only a very small contributing fraction. Scholzen et al. [68] hypothesized that Treg cells might be inducible by the self-regulating FoxP3-Th1 IL-10-producing cells, a mechanism also observed in *L. major*-infection. TGF-β, other major suppressive cytokine is mainly secreted by nTreg cells [69] and macrophages [70]. nTreg cells have been shown to require DC TLR-9 stimulation to be activated [71], but the role of this type of receptors remains controversial, probably due to single nucleotide polymorphisms (SNP) in pattern recognition receptors (PRR) such as TLR, which result in different

degrees of response [59]. TGF-β, IL-10 and IL-2 induce peripheral $CD4^+CD25^-$ T cells to express FoxP3 [66], through antigen presentation by immature DCs [65, 72] and monocytes [73]. It has been shown, however, that direct cell contact is not always necessary for activation of FoxP3 [73], which can gain regulatory function by stimulation from the circulating cytokines IL-2 and IL-10. There is a puzzling nomenclature for Treg cells in malaria, a problem which should be addressed, as it does not facilitate elucidative comparisons nor conclusions between studies.

As with leishmaniasis, it is the balance between effector T cells and Treg cells that determines the severity of infection [68, 74]. Treg cells, while preventing excess inflammation and tissue destruction, can also inhibit the effector function of $CD4^+$ and $CD8^+$ T cells, NKT and NK cells [65], resulting in prevalence of the parasite and disease [75]. Moreover, IL-2 activated-Treg cells express high levels of B-cell lymphoma 2 (Bcl-2), which in turn prevents apoptosis. Competition for IL-2 by Treg cells and memory T cells might be responsible for the dimension of memory cell population, delineating the severity of the symptoms [68]. Literature also indicates that in malaria infection nTreg cells possibly modulate both acute pro-inflammatory and Th1 memory responses [65].

It appears that the cause for different manifestations of pathology is dependent on the levels of key cytokines present at specific stages of infection [58, 66]. If Treg cells are preferentially activated in the early stages of infection severe malaria is more likely to occur [74]; whereas if Treg cells are only activated in the latter stages of infection, effector mechanisms are able to eliminate more infected RBC, resulting in lower parasitaemias and suppressing inflammation at later stage [60]. In the last case, severe forms of the disease are less likely to occur.

$CD4^+CD25^+$ Treg cells have been shown to lower IFN-γ production [76]. Depletion of $CD25^{high}$ cells, of which the majority are Treg cells, protects mice from experimental cerebral malaria, whereas depletion of $FoxP3^+$ cells did not, suggesting that Treg cells may suppress T cell responses of the host [77]. It is not the depletion of Treg cells the sole responsible for protection against cerebral malaria, probably due to the prevalence of $CD25l^{ow}$ $FoxP3^+$ T cells that are retained even after treatment with anti-CD25 mAb. Cerebral malaria is inhibited by suppression of $CD4^+$ cells by $CD4^+CD25^+$ Treg in mice infected with *P. berghei* [78]. Furthermore, they inhibit Th1 memory cells in a secondary challenge. However, it was shown that depletion of Treg cells resulted in increased effector T cell response against circumsporozoite protein in the liver stage, but failed to increase lasting memory [79]. In fact, the only individuals who seem to develop immune memory to *Plasmodium* infection are the ones living in endemic regions where they are repeatedly bitten [67, 80].

More recently, Keswani & Bhattacharyya [81] have shown that Th17 cells which requires both TGF-β and IL-6 to differentiate, might play an important role in disease progression. IL-6 together with lower levels of TGF-β inhibits the proliferation of $CD4^+CD25^+FoxP3^+$ iTreg cells, promoting differentiation of Th17 [81, 82]. During acute infection $FoxP3^+$ Treg cells might be overwhelmed where IFN-γ and IL-10 co-secreting $CD4^+$ effector cells likely auto-regulate themselves; however, if the parasite load is controlled, the Bcl-2 production decreases and $FoxP3^+$ Treg cells suffer apoptosis [73].

D. Babesiosis

Babesiosis is an emerging zoonotic disease caused by the intraerythrocytic protozoan from the genus *Babesia* [17]. This parasite has a worldwide distribution and is transmitted by ticks. *B. microti* is a major cause of a malaria-like illness in humans in several regions of North America and Europe [83, 84]. Its life cycle occurs between the white-footed field mouse, *Peromyscus leucopus*, and the deer tick *Ixodes scapularis*. Humans enter the cycle when bitten by infected ticks [85]. In Europe, *B. divergens* infections have been steadily increasing since the first report in 1957 [86-88]. Among other causes, the increase of human babesiosis cases reflects the augmented population of immunocompromised individuals susceptible to infection, to which *Babesia* infection is often lethal [89].

Babesia spp. infection starts when a tick feeds on a vertebrate host injecting sporozoites together with saliva. The sporozoites penetrate directly the RBC and by binary fission develop into merozoites that, after cell lysis, are released into the blood stream. Each merozoite is able to infect a new RBC and their size and location vary according to the *Babesia* and the host species. When a competent tick takes up a blood meal from an infected host, ingests babesia-infected RBC [89]. Some of these parasites will develop into gametocytes or strahlenkorper (‘ray bodies’) and fuse in the lumen of the digestive track originating zygotes [90]. The zygotes penetrate the midgut cells and transform into a motile stage, the ookinete. This stage migrates from the midgut and invades other tissues, including the ovaries resulting in transovarial transmission. After this point, *Babesia* will develop by asexual multiplication (sporogony), differentiate sporokinetes that invade the salivary glands, developing into sporozoites, the infectious stage for the vertebrate host [91].

The mechanisms of immunity to *Babesia* parasites are hypothesized to require both innate and adaptive immune responses; however, the cell-mediated immune response is considered the most important in reducing the multiplication of *Babesia* within the vertebrate host and, at the same time, probably leading to pathology [92]. To our best knowledge, only one study suggests a possible role of the $CD4^+CD25^+$ Treg cells during *Babesia* infection, what contrasts with the information available for other protozoan parasites aforementioned. Jeong and colleagues [84] have shown that Treg cells, in particular the $CD4^+CD25^+FoxP3^+$ subset, expand during the acute phase of disease in the spleen of *B. microti*-infected mice at 7 and 11 days post-infection, in comparison with control mice, facilitating the growth and survival of the parasite.

In the future, it would be interesting to investigate and clarify the role of the $CD4^+CD25^+$ Treg cells during the infection caused by *Babesia* spp.

IV. Conclusions

Infection by parasitic protozoan still represents major threats to human health worldwide, and the chronic phase of the diseases seems to be balanced in a tolerant state by Treg cells.

This work has given an overview about the function of Treg cells and highlighted the different regulatory mechanisms by which Treg cells may influence the homeostasis of the immune response, avoiding excessive immunopathology during protozoan infections. Amongst all the Treg subsets, $CD4^+CD25^+$ Treg cells are the best characterized to date. However, there is considerable controversy regarding the characteristics and functions of these cells when induced by protozoan infections, and there is no doubt that remains much to be learn for which more research must be undertaken. In the future, more could be gleaned from the long-term interaction between the mechanisms of immune regulation and the parasites. Several reports describe the involvement of $CD4^+CD25^+$ Treg cells with the development and outcome of trypanosomiasis, leishmaniasis, malaria and babesiosis and the persistence of the respective parasites within the host.

With an increasing number of immunocompromised individuals, the current insufficient medical treatments and ineffective prophylaxis alternatives, these infections have become of extreme importance. Great attention has also to be given to healthy immunocompetent individuals, as these protozoan infections are normally associated with the host's inability to clear chronic infection, thus acting as possible reservoirs and contributing for the endemic populations. Any situation that will compromise the immune system can trigger disease reactivation and develop acute and symptomatic stages. Therefore, there is an urgent need to implement efficient strategies to prevent diseases caused by protozoan parasites.

The understanding of these complex interactions would favor the design of immunomodulator drugs or vaccines and contribute for the development of novel of prophylactic and therapeutic strategies.

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