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Humoral immunity in leishmaniasis – Prevention or promotion of parasite growth?

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ABSTRACT

Leishmaniasis can present as a "spectrum" of clinical outcomes. There is evidence that these divergent clinical outcomes are attributable to genetic differences in the human host [1] as well the species of infecting parasite [2]. The spectrum of disease has largely been described by defining the polar opposites of T cell immune responses. In the mouse model, a T_H1 immune response is associated with low numbers of *Leishmania* parasites in lesions, whereas a T_H2 immune response has been associated with unrestricted parasite growth. In the present work, we revisit leishmaniasis and seek to better define the clinical spectrum as a function of divergent humoral immune responses. We describe examples in human, canine, and even some murine models of leishmaniasis that reveal a direct correlation between high anti-parasite antibody responses and unrestricted parasite growth. Therefore, we propose that the spectral nature of this disease may be due to quantitative and qualitative differences in the antibodies that are produced during disease. In human visceral leishmaniasis, a decrease in anti-parasite antibody levels may actually predict disease resolution. Thus, rather than defining this disease as a simple T_H1/T_H2 dichotomy, we propose that clinical leishmaniasis depends on the degree of humoral immunity, with high IgG predicting parasite persistence. These observations have obvious implications for vaccine development in leishmaniasis, and they may extend to other diseases caused by intracellular pathogens.

Leishmaniasis and *Leishmania* spp. Leishmaniasis is a group of diseases, caused by more than 20 *Leishmania* parasite species. These diseases are prevalent in tropical and subtropical countries on four continents, and they present with a wide spectrum of clinical manifestations [1–3]. They are all considered to be neglected diseases that primarily affect poor inhabitants in countries with inadequate social well-being. Because the clinical presentation of leishmaniasis can vary as a function of the infecting parasite species, we start with a brief review of clinical leishmaniasis as well as the parasites causing these disease manifestations. For more thorough descriptions of clinical leishmaniasis, the reader is referred to several excellent reviews [2,4,5]. In the simplest classification system, there are three general forms of this disease, based largely on where the parasites reside and cause pathology. These forms are cutaneous, visceral, and mucosal. In the cutaneous form of the disease, parasites reside in the skin, eventually causing a skin ulceration, typically at the site of the sand fly bite. This is the most common form of the disease and is considered the least pathogenic because in most cases cellular immunity gradually develops to limit parasite growth, replacing the ulcer with a noticeable scar. Cutaneous leishmaniasis can be caused by a number of different *Leishmania* species, including *L. major*, *L. mexicana*, *L. amazonensis*, or *L. braziliensis*. A rare form of cutaneous leishmaniasis is the disseminated form, in which patients infected with *L. braziliensis* or *L. amazonenis*, may have a large number of lesions spread throughout the body. This form should not be confused with diffuse cutaneous leishmaniasis (DCL). In DCL, there is the formation of non-ulcerated and nodular lesions with a large number of parasites within macrophages, whereas disseminated leishmaniasis has ulcerated lesions and relatively few parasites. The two diseases also appear to be immunologically distinct [6]. Mucosal leishmaniasis is a rare form of the disease which can develop slowly after infection with *L. braziliensis, L. guyanenensis*, *L. panamanensis* and less frequently with *L. amazonensis*, in the new world, or by *L. donovani* complex species, mainly *L. infantum* in the old world [7]. Parasite speciation can be difficult in this form of the disease due to the low number of parasites present in the mucosa. Clinical presentation can vary, according to the causative species and individual responses,

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but generally are related to ulcerations of upper respiratory tract, nose or oral mucosa. This disease can be remarkably disfiguring, despite the relatively low numbers of parasites in the lesion. Visceral leishmaniasis (VL) can be caused by *L. donovani* in Asia and Africa and *L. infantum* in the Mediterranean Basin [8]. *L. chagasi* is the species responsible for VL in the new world. In this form of the disease, parasites replicate in the visceral organs including the liver and spleen. If untreated, this form of the disease is associated with a high degree of mortality [9].

In this review, the spectrum of disease will be described not as a function of where the parasites reside, but rather as a function of the immune response each parasite elicits, and the resulting clearance or propagation of parasite numbers in the host. On one end of this immunological spectrum are the species that are efficiently cleared by the host immune response. This form of the disease is associated with the development of a cell mediated immune response (so-called T_H1), which can be visualized by a positive delayed type hypersensitivity (DTH) response in skin to parasite antigens (Montenegro test). On the other end of this spectrum are the organisms that fail to induce an effective immune response. As a result, parasites seem to replicate unchecked in tissue. These diseases have sometimes been referred to as "anergic" forms of the disease, primarily because they are associated with a negative DTH response to parasite antigen. The word anergic in this context may be misleading, because this term implies that the host fails to respond to parasite antigens. In this chapter, we present evidence that the host makes a brisk immunological response to these parasite antigens, but this response is not the appropriate type of immune response, and therefore parasites survive and multiply unchecked in tissue. Therefore, in this disease, rather than a failure to mount an immune response, the host "deviates" to the wrong immune response.

Control of leishmaniasis. Most of what we know about the immune response to this parasite was originally gleaned from seminal studies on different species of mice that were experimentally infected with *Leishmania major* (for an excellent early review, see [10]). Unfortunately, while these models provided much needed information about the murine immune response to this parasite, they left some critical aspects of the human immune response unanswered. In these early studies, there was a clear and well-justified focus on T cell mediated immunity. The T_H1/T_H2 dichotomy, which was essentially characterized in leishmania-infected mice, was thought to be the key to understanding the development of treatments and vaccines against leishmaniasis [11]. This oversimplification of immune responses, though appropriate for some *Leishmania* species, has introduced new challenges to our understanding of the pathogenesis of this disease in humans.

Briefly, murine models of infection have helped to identify resistant and susceptible species of mice based on the prevalence of a T_H1 or T_H2 immune response, respectively [12,13]. The activation state of CD4+ helper T cells in murine models relies heavily on the cytokines IL-12 (T_H1) or IL-4 (T_H2). T_H1 cells produce IFNγ and TNF, molecules that activate macrophages to kill intracellular parasites [14–16]. "Improper" T_H2 responses, generated via the production of IL-4 and IL-13 signaling through IL-4 receptor-alpha result in alternative macrophage activation, defective macrophage-mediated killing, and survival of parasites in susceptible mice [17]. Susceptibility in this model has also been tied to anti-inflammatory IL-10 production as well. Mice deficient in IL-10 are highly resistant to infection from multiple species [18,19]. This brief description of the mouse model admittedly ignores several important subtleties of this infection model. For example, the mixing T_H1/T_H2 immune responses seems to help susceptibility. Furthermore, while there is no doubt about the participation of T lymphocytes in the resistance to disease [20,21], in the absence of T lymphocytes, the development of lesions in mice can be variable, and depend on the infecting *Leishmania* strain as well [22].

In human leishmaniasis, the protective aspects of IFNγ production by T_H1 cells that were observed in murine models of parasite clearance have largely been recapitulated. However, these same high levels of IFNγ and TNF appear to drive immunopathology in localized and mucocutaneous infections caused by *L. braziliensis* [23,24]. The association of IL-4 production with susceptibility to human disease has been less consistently documented, with some studies indicating, that IL-4 contributes to susceptibility [25–27], but not others [28,29]. The relatively high levels of IL-10 and TGFβ [30,31] in human cutaneous leishmaniasis lesions suggest a more complicated scenario where other cytokines can also contribute to parasite persistence.

The treatment of human leishmaniasis varies with the clinical presentation of disease. Simple cutaneous leishmaniasis will eventually heal, but this form of the disease should always be treated to accelerate cure and avoid complications, such as dissemination. Visceral leishmaniasis, on the other end of the clinical spectrum, requires aggressive treatment because untreated disease is associated with a high degree of mortality. The mucosal or diffuse cutaneous forms of leishmaniasis are more challenging to treat, and for these disease forms, the T_H2 dichotomy that was applied to progressing murine infections simply does not apply. While the chemotherapy of leishmaniasis has gradually improved, treatment of these later forms of the disease continues to be a challenge and the drugs remain expensive, toxic, and only moderately effective.

Given these limitations to chemotherapy and the fact that patients who recover from the disease generally become resistant to re-infection, a strategy to vaccinate against leishmaniasis seems all too logical [32,33]. However, the type of vaccine to be used and the type of immunity this vaccine elicits may not be quite as obvious. The history of vaccines against leishmaniasis is largely a reflection of the history of vaccination itself. Leishmanization (variolation-like immunization) was a primitive way to offer protection against natural infection by using live parasites injected directly under the skin. Although largely effective, the risk of using virulent parasites, prevented its widespread use [34]. The first trials using killed or attenuated vaccines against leishmaniasis were undertaken in the early 1940s, but these attenuated/killed parasites provided only limited protection [35]. Since then only episodic progress in the development of an effective vaccine against leishmaniasis has been achieved, despite considerable advances in vaccine development in general [36]. Cecilio and colleagues, in 2018, described the different vaccine formulations, most of which have provided modest success in humans and animals [37]. It seems remarkable that after almost 80 years of trials, we still do not have an approved vaccine for human leishmaniasis. For most other diseases, the vaccines that frequently make it to market induce a humoral immune response to purified protein antigens. Many such antigens have been identified and purified from *Leishmania* spp., and several of these antigens have become vaccine candidates. In this review, we seek to raise the very basic question of whether anti-parasite antibodies to defined leishmania antigens will provide the best immune correlates for protection against naturally acquired leishmaniasis.

Immune deviation. The term immune deviation has been used loosely throughout the literature to describe many different and unrelated immunological phenomenon. In this chapter, we use it as it was originally defined to describe the inverse correlation between the production of antibody and the establishment of cell mediated immunity. In 1965, Asherson and Stone first observed a reduction in delayed type hypersensitivity in guinea pigs by previously injecting them with a different formulation of the same antigen prior to the skin test [38]. They termed this phenomenon "immune deviation", but were unable to determine the mechanism of this unexpected observation [39]. Some 10 years later, working in the anterior chamber of the eye, Kaplan and Streilein observed that the appearance of antibody coincided with the suppression of cell-mediated immunity [40] and proposed this as a mechanism behind immune deviation. Coincident with these observations was a series of studies undertaken by Parish and colleagues [41], which demonstrated an inverse correlation between DTH responses and antibody formation [42]. Jerry and colleagues observed high levels of circulating immune complexes in anergic melanoma patients and called

this “deranged immune regulation” [43]. As early as 1982 Sehgal and Pathania, observed high levels of immune complexes in the serum of patients with Indian kala-azar (visceral leishmaniasis), and speculated that these high antibody levels might consume complement and depress cell mediated immunity [44]. Working in the murine schistosome model, Colley and colleagues demonstrated that the administration of high doses of schistosome egg antigen (SEA) induced high levels of anti-SEA antibody levels but depressed cell-mediated immune responses [45]. Thus, in many divergent infectious, neoplastic and immunological disease models, the inverse correlation between Cell-Mediate Immunity (CMI) and humoral immunity was established - and largely forgotten.

Immune deviation and parasite persistence. The goal of this review is to apply the concept of immune deviation to leishmaniasis, and address the topic of whether anti-parasite antibody is protective. We present evidence in experimental mouse models of disease, in canine leishmaniasis, and from human visceral and cutaneous disease where high antibody levels not only fail to provide protection to the host, but are actually a strong predictor of parasite persistence. If this is indeed the case, then current vaccine approaches may need to be reconsidered for this intracellular parasite.

In the mouse model of infection with *L. major*, several seminal studies associated progressive disease in BALB/c mice with a non-protective T_H2 immune response, whereas a T_H1 response promoted disease resolution (reviewed in [10]). Several years later, we (and many others) confirmed the susceptibility of BALB/c mice to this infection, but we demonstrated that JH mice [46] lacking B cells and immunoglobulins on the BALB/c background were surprisingly resistant to disease [47]. Lesions were much smaller with 4 logs fewer parasites in them. Importantly, if we added back anti-parasite antibody to these resistant mice, they paradoxically became more susceptible, developing lesions as large as wild-type BALB/c mice with similar high levels of parasites in them [47]. Thus, in our hands working in the experimental model of *L. major* infection of susceptible BALB/c mice, high antibody levels proved to be detrimental to host defense. Similar results were obtained by Kima and colleagues, who demonstrated that mice deficient in FcγR signaling were refractory to *L. amazonensis* infection [48]. Buxbaum and Scott subsequently demonstrated that *L. Mexicana* interactions with macrophage FcγR induced the production of IL-10 to exacerbate disease [49]. Therefore, in three different mouse models of leishmaniasis, caused by three different species of parasites, the presence of high levels of anti-parasite antibodies were (surprisingly) associated with increased disease severity. These observations are not restricted to mouse models of infection. Gamma-globulin levels have been shown to be increased in infected humans and in naturally or experimentally infected canine species [50–52]. Furthermore, hypergammaglobulinemia can be a hematological finding used to predict disease progression [53,54].

Working on visceral leishmaniasis with our collaborator Dr. Selma Jeronimo in Natal-RN, Brazil, we demonstrated that patients with high levels of anti-parasite antibodies were more likely to express a defective Delayed Type Hypersensitivity (DTH) response, resulting in reduced skin test responses to parasite antigens [47]. Furthermore, after these patients received anti-parasite chemotherapy, their antibody levels declined as their DTH responses were restored. Positive patient DTH responses have long been associated with a healing response in this form of leishmaniasis [55], consistent with Th1 cells promoting parasite elimination. The lack of DTH responses along with high IgG responses have been associated with disease progression [56]. The observations we made in Brazilian patients with VL are consistent with studies showing high antibody seropositivity in patients with Indian kala-azar [57] and in dogs with visceral leishmaniasis [58].

Our analysis of lesion transcriptomes from patients with different forms of tegumentary leishmaniasis provided the strongest associations between high antibody levels and parasite persistence. Working with our collaborators, Drs. Phillip Scott and Edgar Carvalho in Salvador-BA, Brazil, we performed RNA-sequencing to quantify antibody transcript levels in the skin of patients with self-healing (so-called “localized”) cutaneous leishmaniasis (LCL) caused by *L. braziliensis* [59]. In the localized form of this disease, parasite numbers are typically quite low, despite considerable immunopathology [60]. Lesion biopsy material from 25 patients with LCL revealed relatively low numbers of transcripts typically associated with B cells (eg. CD19, CD20, CD79), and similarly low expression of transcripts encoding host IgG fragments [59]. One of the advantages to RNA-sequencing is that lesion transcripts can be confidently mapped to either the host or the parasite. This allows one to correlate the levels of host immunoglobulin transcripts with the relative quantity of parasite transcripts in the lesion. In this localized form of the disease, all 25 of the patients analyzed had relatively low amounts of parasite transcripts in their lesions, with less than 1.5% of the total lesion reads mapping to the parasite genome (the rest mapping to the host genome). In fact 10/25 patients had undetectable levels of parasite transcripts. But six of the 25 patients analyzed had slightly higher levels of parasite transcripts, ranging between 0.4 and 1.4% of the total detectible transcripts. This small subset of patients with modest elevations of parasite transcripts, expressed higher levels of transcripts encoding B cell markers and higher levels of immunoglobulin transcripts in their lesions. In fact 8 of the 10 most highly upregulated transcripts in these lesions encoded immunoglobulin gene fragments [59]. The 10 patients with undetectable parasite transcripts expressed low to undetectable levels of B cell and immunoglobulin transcripts. Thus, in localized cutaneous leishmaniasis cause by *L. braziliansis*, there was a direct correlation between parasite transcript numbers and host immunoglobulin transcript frequency in lesions.

To further test the association between parasite persistence and immunoglobulin levels, we reached out to Dr. Fernando Silveira in Belem, Para, Brazil to study 6 of his patients with a rare form of diffuse cutaneous leishmaniasis (DCL) [61]. Patients with the diffuse form of the disease have numerous lesions across the dermis with large numbers of parasites in them. By RNA-seq, the percentage of transcripts that mapped to parasites in DCL lesions ranged (remarkably) from 10 to 28%, consistent with microscopic observations that revealed numerous macrophages infected with large numbers of parasites. When the host transcriptome was analyzed in this progressive disease, 38 of the 40 most highly upregulated transcripts (relative to uninfected controls) encoded B cell or immunoglobulin genes. The amount of immunoglobulin transcripts in these lesions was quite remarkable. The 10 most highly upregulated host transcripts were immunoglobulin transcripts, and all were above 1000 RPKM (reads per kilobase of transcript per million mapped reads) in all six patients [61]. Therefore, in this progressive form of the disease where parasites persist unchecked in lesions across the skin, the parasites are swimming in a pool of IgG that does not appear to provide protection. When all 31 patients with cutaneous leishmaniasis (localized and diffuse) were grouped together and analyzed, we see a direct correlation between the abundance of parasite transcripts in lesions (defined as a percentage of total reads) with the levels of host cell transcripts encoding IgG (constant and variable regions) (Fig. 1).

Macrophage phenotypes in cutaneous leishmaniasis. The failure of macrophages to eradicate parasites in DCL lesions, and the high levels of host IgG in these lesions prompted us to examine macrophage phenotypes in cutaneous leishmaniasis. We had previously published studies with murine macrophages that were stimulated in vitro with various TLR (toll-like receptor) ligands in the presence of immune complexes (IC) [62–65]. Stimulation with TLR ligands alone resulted in the expected inflammatory macrophage (so-called M1) response. However, the combination of two stimuli, TLR ligands plus IC, yielded a fundamentally different cellular phenotype. Macrophages stimulated in this way produced myriad growth and angiogenic factors, the anti-inflammatory cytokine IL-10, and reduced inflammatory transcripts. We tentatively termed these cells regulatory macrophages, because their main function appeared to be the maintenance of homeostasis and the restoration of tissue to its non-inflamed state [66].

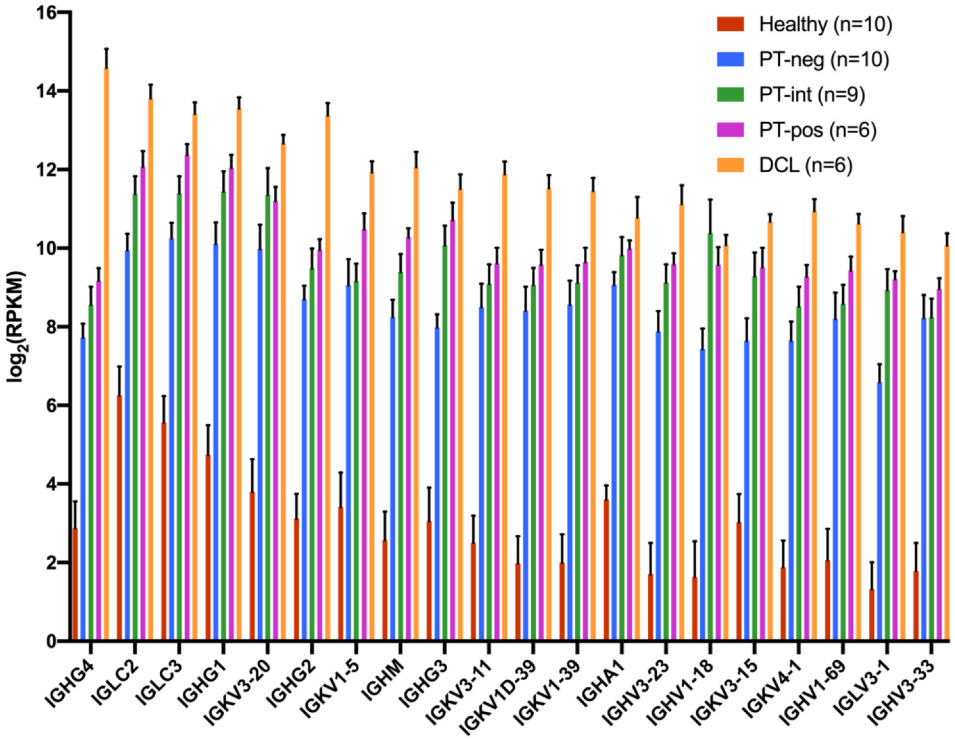

Fig. 1. (A) Human host immunoglobulin transcripts correlate with *Leishmania* parasite transcript abundance in lesions. A bar plot (mean + SEM) of the top 20 immunoglobulin transcripts (X-axis) by average reads per kilobase per million reads (RPKM) and separated into healthy skin (n = 10), patient samples containing *L. braziliensis* transcripts mapping at < 0.01% of the total reads in the lesion metatranscriptome (Parasite transcript-negative, n = 10), 0.01–0.5% (Parasite transcript-intermediate, n = 9), 0.5–2.0% (Parasite transcript-pos, n = 6), or patient samples from DCL (n = 6) with > 10% of the transcripts mapping to *L. amazonensis*. Linear regression between parasite transcript abundance and immunoglobulin gene expression showed significant correlation for 19 of the top 20 (all but IGHV1-18) and 67% of all captured immunoglobulin transcripts (106/159).

We subsequently examined the transcriptomes of human macrophages, stimulated in vitro with TLR ligands along with immune complexes [67]. This analysis allowed us to identify transcripts and proteins associated with this regulatory phenotype. These macrophages show reduced expression of inflammatory chemokines, including CCL8, CXCL9, CXCL10, and CXCL11, reduced levels of antimicrobial effectors, including NOS2 and indolamine dioxygenase (IDO, tryptophan catabolism), and decreased inflammatory cytokines, including IL-1, IL-6, and IL-12. They also upregulated a variety of matrix metalloproteases, the anti-inflammatory cytokine IL-10, and two surface biomarkers, DC-STAMP and MARCO.

Macrophages transcripts from lesions from patients with DCL were compared to macrophages from LCL lesions, to determine whether differences in the phenotype of macrophages could predict parasite persistence (DCL) or eradication (LCL). This analysis was possible because despite the fact that the two lesions looked macroscopically quite distinct and had large differences in the number of parasites within them, the number of macrophages in LCL and DCL appeared to be quite similar. There were no differences in transcript numbers for FcγR, CD11b, CD18, CD68, or the mannose receptor (CD204). Macrophages in localized cutaneous leishmaniasis, caused by *L. braziliensis*, exhibited a classical activation phenotype and produced higher levels of transcripts for TNF, NOS2, a variety of inflammatory chemokines, including CCL8, CXCL9, 10, and 11, and high levels of IL-6 [61]. All of these transcripts are associated with inflammatory (M1) macrophages, and driven by T_H1 T cell responses. These macrophages promote the immunopathology that has been associated with this disease [60]. These inflammatory-associated transcripts were significantly reduced in DCL relative to LCL. In fact, transcripts for IL-6, NOS2 and CXCL11 were undetected in DCL lesions, suggesting that macrophages in the Ig-rich environment of DCL fail to express important inflammatory effector molecules (see Graphical Abstract). When we examined transcripts that were upregulated in DCL, relative to LCL, several regulatory transcripts, including the matrix metalloproteases MMP11 and MMP19, DC-STAMP and MARCO were all elevated. Thus, macrophages in human DCL assume a regulatory phenotype.

Status of vaccines for human and canine Leishmaniasis. Although vaccines for human leishmaniasis remain a challenge, there is reason for optimism. Early studies in Brazil [68], utilized a mixture of killed promastigotes in a phenol-saline solution, isolated from patients with a variety of different forms of cutaneous leishmaniasis. They were based on the results obtained in a trial by Dr. Salles-Gomes (1939) that used a suspension of dead promastigotes, which were intravenously inoculated in patients with American Tegumentary Leishmaniasis (ATL). After intravenous injection of the suspension they observed a therapeutic effect, characterized by the reduction in the size of the lesions in the patient as the treatment progressed [69]. Similar efficacy of iv vaccination of mice has also been reported [70]. Indeed, this iv route of vaccination was recently shown to be particularly effective in providing immunity against intracellular pathogens [71]. The mechanisms for this protection have not yet been definitively established, but in the case of leishmaniasis the administration of multiple parasite antigens into the blood may result in the “capture” of circulating anti-parasite antibody complexes via CR1 expressed on red blood cells [72]. This depletion of anti-parasite antibody may skew the immune response towards cell mediated immunity. The lack of an effective vaccine for human leishmaniasis is consistent with the idea that the induction of humoral immunity, to the exclusion of cellular immunity, will not provide protection. Three vaccines for canine visceral leishmaniasis are commercially available in Brazil and Europe. All three vaccines have in common the ability to induce a robust cellular immune response, with the production of high levels of IFN-γ and in some cases, low levels of IL-4 [73,74]. Although the canine vaccines induce anti-parasite antibodies [75], they do not induce the hypergammaglobulinemia that is observed in dogs with the naturally occurring disease.

Therefore, these studies begin to identify immune correlates to protection of a successful human vaccine for leishmaniasis. These immune correlates would be centered on T cell activation, with robust IFN-γ production and no interference due to T_H2 immune responses. While this vaccine would logically induce anti-parasite antibody, the effectiveness of this vaccine would not correlate with antibody levels, because excessive levels of anti-parasite antibody may actually predict a failed vaccine. Finally, a single human vaccine for all *Leishmania* species may not be feasible, because it would have to provide protection across

the entire immunological spectrum that is observed in the various "spectral" diseases that encompass human leishmaniasis.

Author statement

All authors contributed equally to the conceptualization and writing of this review.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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