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Immunoregulation in Human American Leishmaniasis: Balancing Pathology and Protection

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Summary

Leishmaniasis covers a broad spectrum of diseases with distinct, and sometimes overlapping, characteristics. The common thread in all forms of leishmaniasis is the infection by the parasite *Leishmania* belonging to the genus *Leishmania*. Upon infection of humans there can be at least three outcomes, 1) control of *Leishmania* by the host immune response resulting in asymptomatic disease, 2) patent infection and development of a relatively mild form of leishmaniasis, and 3) patent infection and development of severe clinical forms. The factors that determine the outcome of an initial inoculation with *Leishmania* are many, with the species of *Leishmania* representing one of the strongest predictive factors for the development of a given clinical form of disease. This is seen with *L. braziliensis* and *L. amazonensis*, infection leading mostly to tegumentary forms of disease, and *L. infantum* with the potential to induce visceral disease. However, it is also clear that the host immune response is a key factor in disease progression, not only responsible for control of *Leishmania*, but also playing an important role in disease progression and pathology. This duality between protective and pathogenic immune responses in individuals infected with *Leishmania* in the Americas is the focus of this review.

Initial host-parasite interaction: disease development vs. asymptomatic exposure

Natural infection via a *Leishmania* contaminated sand-fly bite can lead to asymptomatic exposure or development of severe leishmaniasis in humans (1, 2). This first decision point holds for infection with species that eventually lead to either tegumentary or visceral disease (1). Upon natural infection, *Leishmania* metacyclic promastigotes gain access to the host where they encounter neutrophils, macrophages and monocytes, all of which can serve as

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targets for infection (2–5). It has become clear over the years that all three cell types can be infected by *Leishmania* and that the proportion of each cell type infected, the microenvironment present at the site of infection, the activation state of the cells, and the intensity of infection, all culminate in the development of a productive *Leishmania* infection, or possibly extinguish *Leishmania* before it progresses to a patent infection (Figure 1). Upon infection of host cells, *Leishmania* is likely shuttled to the nearest draining lymph nodes where again, depending on the microenvironment and cell types most involved, further replication and dissemination of the infection can occur, as well as the development of the adaptive immune response made up of B and T cells (CD4, CD8, double negative) (1, 2). Thus, these stages are the earliest events that determine whether an initial inoculation with *Leishmania* will go on to a form a patent infection, or be halted at this early stage.

Interaction between *Leishmania* and a number of potential surface receptors expressed by host neutrophils, dendritic cells, macrophages and monocytes trigger phagocytic activities, allowing *Leishmania* to gain access into these cells (6–11). It was recently shown that infection of neutrophils can lead to *Leishmania* death, but also to the generation of apoptotic bodies that are capable of inhibiting effective responses of nearby macrophages (4). This initial contact can influence the replication of *Leishmania* in the critical early phases of infection. If macrophages are infected, there are also a variety of responses that can be triggered depending on the macrophage activation state and the species of *Leishmania* (2, 12–15). In addition to controlling parasite replication or not, the macrophage can also produce highly active immunoregulatory cytokines, such as the inflammatory cytokines TNF-alpha and IL-1 and the down modulatory cytokines, TGF-beta and IL-10 (2, 12, 16–20). Again, which cytokines and biological activities expressed by the infected macrophage depend on the activation state of the cell, the *Leishmania* species or strain, the sand-fly vector saliva activities present, and host genetics (1, 2, 21). Recent studies have shown that strain differences within the same species are associated with a given clinical form of disease (22, 23). Several studies have shown the importance of host genetic makeup in susceptibility to tegumentary and visceral forms of leishmaniasis (2, 24–30). These host genetic differences can play important roles in determining whether an individual will induce an effective leishmaniacidal response or not, as well as determine the further development of disease and pathology. Many gene polymorphisms identified as susceptibility factors for leishmaniasis are in genes that code for molecules associated with the immune response, especially cytokines and HLA alleles (25, 28, 31). One can postulate, for example, (32) that an individual with a high producer allele for TNF-alpha could be primed to respond more effectively with leishmaniacidal activity of infected macrophages early following infection. However, if the initial infection is not controlled, that same high production of TNF-alpha could later contribute to more severe immunopathology development as seen in mucosal leishmaniasis (30, 33–35).

Interestingly, different monocyte and macrophage profiles have been associated with distinct clinical forms of leishmaniasis. The majority of the studies have been performed when an active adaptive immune response is already induced in the infected individual and, thus, the host monocytes and macrophages are already in an environment dominated by inflammatory

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cytokines and active CD4+ T cells, DN T cells and CD8+ T cells, all producing bioactive cytokines and chemokines (2, 36–38). Nevertheless, clear differences are seen in monocytes and macrophages isolated from leishmaniasis patients as compared to non-infected controls and comparing between clinical forms of disease (39–41).

Thus, the initial *Leishmania*–host interaction culminates in either the control, with or without "sterilization," of *Leishmania* likely leading to an asymptomatic non-productive infection, or it leads to a patent *Leishmania* infection expressed as either tegumentary diseases, such as cutaneous, mucosal or disseminated clinical forms (associated with *L. amazonensis, L. braziliensis, L. guyanesis, L. mexicana* and others in the Americas) of leishmaniasis, or visceral disease (*L. infantum chagasi* in the Americas) (Figure 1).

Pathogen control vs. pathology: immune balancing act in human leishmaniasis

Tegumentary disease

Once an adaptive cellular immune response develops following the presentation of classic peptide antigens by MHC class I for CD8+ T cells, MHC class II for CD4+ T cells, and CD1-presented lipid antigens to DN T cells and invariant NK T cells (iNK T cells), the balance between control of the pathogen and pathology development is amplified (1, 2, 33, 36, 42). The initial expansion of T cell subpopulations with distinct functional potentials begins in earnest within the draining lymph nodes following the initial infection (Figure 2). There, T cells differentiate towards effector T cells following activation through their interaction with antigen presenting cells (APCs). These differentiated T cells, capable of producing distinct cytokines can home to infection sites and drive efficient anti-*Leishmania* immune responses (Figure 2) both at the lesion site and in draining lymph nodes. These responses are greatly driven by Th1 CD4+ T cells producing monocyte and macrophage activating cytokines like IFN-gamma and TNF-alpha, together with regulatory cytokines like IL-10 (1, 2, 38). Importantly, the balance begins between controlling *Leishmania* replication within infected macrophages and monocytes, and the induction of immunopathology by excessive inflammatory responses (33, 35, 42–44). Activated Th1 cytokine producing CD4+ T cells have been correlated both with effective leishmaniacidal immune responses, as well as with greater pathology in cutaneous disease (35,45), and in mucosal disease, where an exacerbated Th1 type CD4+ T cell response is present (32–34, 46–49). Moreover, CD4+ T cells expressing the TCR V beta region, Vbeta5, have been associated with lesion size and differential lesion homing, indicating the possible role of these cells in pathology (44). In addition, the strong inflammatory CD4+ T cell response seen in cutaneous leishmaniasis (CL) is accompanied by increased IL-10 producing T cells (43), and the same is seen within the monocyte population (32). Thus, an ongoing regulation of the inflammatory response in CL disease is apparent, while that same co-regulation is faulty in mucosal leishmaniasis (ML) (32). Interestingly, while TNF-alpha and IFN-gamma are associated with the eventual resolution of disease in CL, lower levels of these cytokines are associated with disseminated leishmaniasis, indicating a weak cell mediated T cell response and thus, poor control of *Leishmania*, but also a lack of tissue destruction as seen in cutaneous and mucosal disease (50–52), both of which are associated with higher levels

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of TNF-alpha and IFN-gamma. Moreover, monitoring CD4+ T cell activation and cytokine production during active disease and following disease resolution has indicated their important role in finalization of disease and pathology (49, 53, 54). Recently, a correlation between IL-17 production and tegumentary and mucosal disease was found (46, 55). Thus, the nature of the CD4+ T cell response during active CL and through disease resolution is associated with robust production of inflammatory cytokines such as TNF-alpha and IFN-gamma in the presence of IL-10, a regulatory cytokine that acts to reduce production of TNF-alpha by host macrophages and monocytes (Figure 3) (43). Interestingly, treatment of leishmaniasis patients with pentoxifyline to reduce the activity of TNF-alpha leads to quicker resolution of lesions in both CL and ML (56–60). These groups of studies, and others not cited here, have produced a model of the role of CD4+ T cells in orchestrating the initial leishmaniacidal response, but also for contributing to lesion development in both cutaneous and mucosal disease (Figure 3).

In addition to classic CD4+ T cells producing key regulatory cytokines for induction of leishmaniacidal responses, CD8+ T cells also play a role in both the establishment of the cytokine environment, as well as in cytolytic activity (2, 33). Several studies have shown that CD8+ T cells are associated with both protective immunity in CL, as well as greater pathology in CL and ML (61–65). In addition to cytokine production, which is lower in both frequency and intensity as compared to the CD4+ T cell subpopulation, CD8+ T cells also express cytolytic molecules such as granzyme and perforin. Progression of CL lesion has been associated with an increased frequency of CD8+ T cells expressing granzyme (63). Thus, the role of CD8+ T cells in both control of *Leishmania* and in lesion pathology is likely important.

Finally, a minority subpopulation of highly activated, cytokine producing T cells identified by their lack of both CD4 and CD8 co-receptors, termed double negative (DN) T cells, were shown to be the second most prevalent producers of inflammatory cytokines in active CL (38). This DN T cell population is a heterogenous population made up of T cells that recognize antigens presented by classic MHC molecules, class I and class II, as well as by non-classical presenting molecules, CD1. CD1 molecules have an antigen-binding cleft that is shallow and better suited for presentation of lipid and carbohydrate antigens than peptide antigens (66, 67). However, despite this heterogeneity, the DN T cell subpopulation is greatly committed toward the production of inflammatory cytokines (TNF-alpha and IFN-gamma) and, when subdivided into those T cells expressing the alpha/beta T cell receptor (TCR) vs. those expressing the gamma/delta TCR, one can see a clear division into T cell populations that produce a biased inflammatory environment (alpha/beta DN T cells) vs. those that produce a biased regulatory environment (gamma/delta DN T cells) (37). Thus, these T cell subpopulations may carry out distinct roles in parasite control vs. control of pathology in human CL.

Overall a picture of the duality of the T cell response on the broad view of *Leishmania* control vs. the development of pathology has been identified for the majority of T cell subpopulations studies in human CL and ML. This duality represents a classic immunological concept now clearly demonstrated in a complex human parasitic disease (Figure 3). The ability of immunotherapies to modulate these responses is key toward

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development of novel treatments that can accelerate healing and possibly reduce the toxicity of existing treatments by providing immune therapies that could serve as treatment adjuvants with existing therapies. Such treatment has been demonstrated effective by the use of pentoxifylline to reduce pathology and speed healing during CL and ML (56–60). In addition, the use of immune modulating compounds such as n-acetyl-l-cysteine may act in conjunction with glucantime to produce more effective leishmaniacidal responses while regulating some of the immunopathology induced during infection (68, 69). Further studies are needed to determine the suitability of this approach in human disease.

Visceral disease

Visceral leishmaniasis (VL) presents a case of a misbalanced immune response, but unlike in CL and ML where a strong inflammatory and leishmaniacidal response is poorly controlled leading to the formation of excessive immunopathology, VL is the result of an ineffective leishmaniacidal immune response. This defect leads to the dissemination of *Leishmania* in the host and a generalized immune disbalance dominated by increases in down modulatory cytokines like IL-10 and TGF-beta, both of which dampen the effectiveness of the cellular anti-*Leishmania* response (Figure 4). The reasons why VL can develop following infection with *L. infantum* in the Americas, while typically not following infection with *L. amazonensis* or *L. braziliensis* for example, are many fold and include the initial host-parasite interaction, temperature sensitivity of the parasite, the host immune response under the influence of host genetics and past immune experience, and even the sand fly – host interaction. Thus, the outcome of an estimated 5% of infections with *L. infantum* is VL, which culminates in the dissemination of *Leishmania* throughout the body concentrating within macrophages and monocytes in the bone marrow, liver, and spleen (1, 2, 70).

The immune response in individuals following infection with *L. infantum* can be effective in controlling infection and generating a strong delayed type hypersensitivity response. In these cases, the infected individual is typically asymptomatic and the strong DTH is associated with high levels of IFN-gamma and TNF-alpha produced by CD4+ T cells (70, 71). IFN-gamma is an important cytokine involved with resistance of infection in VL due to its capacity to induce the production of ROS in phagocytic cells, which lead to destruction of the parasite (72).

Evaluation of cytokine levels in active VL caused by *Leishmania donovani and Leishmania infantum* have shown high levels of IFN-gamma in serum (73) and plasma (74–76). However, it is believed that despite the presence of high levels of IFN-gamma during infection, the host may fail to control *Leishmania* and mount an effective response to kill the parasite, partly due to an IFN-gamma receptor blockage in macrophage signaling (74). Other researchers have found a mixed profile of cytokines during active VL, associated with high levels of IFN-gamma, IL-10 and IL-6, demonstrating an exacerbation in immune response in these patients (74, 76). Following treatment, VL patients show a reduction in these cytokines and eventually return to levels similar to those observed in healthy individuals (74, 75). Another inflammatory cytokine associated with active VL is TNF-alpha (77). This cytokine seems to plays an important role in disease progression (77). IL-6, while at high levels

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during active VL before treatment (74, 75), also remains significantly elevated compared with control individuals even after treatment, indicating that it may have some beneficial role in disease resolution (74). Despite the high levels of inflammatory cytokines, increased levels of IL-10 in serum from active VL patients have been associated with severity of VL (75, 78–81) (Figure 4). Several articles have shown the importance of IL-10 in the pathogenesis of VL (82–84). Gautam et al. have shown that IL-10 is related to the chronicity of infection, suggesting a direct decrease in T cell activation and/or an inhibitory effect directly on APC. These studies demonstrated that blocking IL-10 in cultures of splenic asperates from VL patients led to a decrease in the parasite load (83). Thus, it is believed that IL-10 plays an important role in the suppression of the immune response, and thus is an important therapeutic target in VL (83, 84). TGF-beta also seems to be important in the development of VL, given its association with high levels of IL-10 in active VL (85).

Deciphering the mechanisms involved in the regulation of cytokine production during active VL infection as compared to asymptomatic individuals will contribute to a better understanding of the immunological phenomena that occur during disease progression. Immune responses amongst individuals with the subclinical form of VL have shown variable cytokine profiles, predominantly characterized by low levels of IL-10 (76, 77, 86), IL-12 and IFN-gamma (77), suggesting an important role for these cytokines in disease development (86). A follow-up study of patients with active and subclinical disease showed that the levels of IL-10 and TNF-alpha were higher in the acute form of the disease than in subclinical individuals (77). These studies suggest that production of both resistance and susceptibility cytokines may be important for contributing to distinct clinical manifestations (86). Although in recent years research has intensified concerning the role of the cytokine IL-17 in protozoa infection, very little is known about its activities in VL. Studies have indicated that IL-17 may be involved in the pathogenesis of CL (87, 88). Interestingly, the opposite is suggested in Chagas disease, given the association of IL-17 expression with the occurrence of the indeterminate (asymptomatic) clinical form (89). Studies seeking to understand the importance of Th1, Th2 and Th17 responses in *Leishmania donovani* induced VL have found that individuals who did not develop Kalazar (KA), or who were protected against KA during a severe outbreak, produced higher levels of IL-17 and Th1 cytokines than those with active KA, suggesting their role in protection from KA (90).

Effective T cell immunity requires activation and differentiation of T cell subpopulations into active effector cells. This activation and differentiation depends on efficient antigen presentation, as well as co-stimulatory activation via molecules like CD28, which is balanced by inhibitory networks such as those provided by CTLA-4 (91), PD1, and PD1-L (92). Through the analysis of markers associated with anergy/exhaustion in CD8+ T cells, it was demonstrated that VL subjects before treatment had higher expression of CTLA-4 and PD-1, than after treatment, or as compared to control individuals (93). These authors went on to suggest that this inhibitory network limits the ability of the individuals to produce IFN-gamma and effectively combat *Leishmania* (93). In experimental models of VL, blockade of CTLA-4 can result in enhanced host resistance to intracellular pathogens. The administration of monoclonal antibodies anti-CTLA-4 in BALB/c mice infected with *Leishmania donovani* enhanced the frequency of IFN-gamma and IL-4 producing cells in

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both spleen and liver and, thus, indicated a potent immunomodulatory activity of CTLA-4 *in vivo* (94). CTLA-4 suppression is important because of its capacity to mediate TGF-beta production. However, higher production is critically involved in intracellular parasite replication (95). Finally, given CTLA-4's role in maintenance of T cell homeostasis, it seems clear that more research on this molecule's role in human VL is warranted (95).

The Programmed Death 1 (PD-1) receptor: PD Ligand (PD-L) pathway is another major receptor–ligand network that functions primarily to provide an inhibitory signal. The inhibitory receptor PD-1 and its ligand PD-L (B7H1) have been shown to play an important role in T cell regulation (96). Joshi et al. demonstrated the importance of CD8+ T cells in the control of *Leishmania* infection in animal models where CD8+ T cells produce cytokines like IFN-gamma that may aid in parasite killing. However, the PD-1/PD-L interaction seems to induce apoptosis and inhibit proliferation of CD8+ T cells, and thereby reduce *Leishmania* control as seen by recovering T cell activity upon blocking the PD-1/PD-L interaction (96). Figure 4 summarizes the results found above in patients during active VL as compared to the asymptomatic infections.

Several studies have shown the importance of immunoregulatory cytokines described above in experimental mouse models of visceral leishmaniasis and some are discussed breifly in Box 1.

Box 1

Experimental murine models of visceral leishmaniasis

To better understand the role of immnoregulation and immunopathology in VL induced following infection with *Leishmania donovani* and *Leishmania chagasi/infantum*, experimental mouse models have been extensively studied. A study evaluating the immune response in BALB/c mice with an inoculum of different concentrations of *L. chagasi* showed that those mice infected with low doses seemed to respond with the production of IFN-gamma. However, when higher doses where administrated the type of immune response changed and IL-10 prevailed, together with progression of severe disease indicating the importance of immunoregulatory cytokines in disease development in this model (97). The importance of IFN-gamma in macrophage activation and *Leishmania* killing has been studied in BALB/c IFN-gamma knockout mice and highlighted a fundamental role for this cytokine in disease resistance (98). Finally, mice deficient in TNF-alpha have increased susceptibility to hepatic *L. donovani* infection (99, 100).

Studies using mice lacking the gene for IL-10 showed that this cytokine plays a central role in susceptibility to *L. donovani* infection. In the absence of IL-10, mice rapidly developed an enhanced Th1 type response. The consequence of this response is a highly effective control of visceral parasites (101, 102). Experiments blocking IL-10-receptor or using anti-IL-10 monoclonal antibodies showed that the immune response and the production of higher levels of IFN-gamma could be restored, which led to activation of macrophages, and consequently parasite death (102–105). Interestingly, IL-6 knockout mice infected with *L. donovani*, had better control of *Leishmania* in the liver and higher

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IFN-gamma production with effective granuloma formation, thus suggesting that IL-6 played a role in pathology in this model (106). Experiments using anti-TGF-beta mAb did not demonstrate significant effects on IFN-gamma levels, showing that the ability to modulate IFN-gamma levels is clearly a hallmark of IL-10 activity (107, 108).

Pathology resolution and protection: role of memory and pathogen persistence

Once active leishmaniasis is diagnosed treatment typically begins within a week or so after the initial diagnosis, or sooner depending on the clinical form and patient complications (co-infections, other diseases, etc.). Most studies of disease resolution have been performed before and after treatment, with a few during treatment. Many factors determine an individual's response to treatment and disease resolution. As stated above, the adaptive immune response plays important roles in both parasite control and development of pathology. Studies in animal models have suggested that sterilizing immune responses early after infection with *Leishmania* can lead to poor maintenance of memory responses (109). In addition, the development and maintenance of effector memory vs. central memory T cell populations can be influenced by the continuing presence of *Leishmania*, even after disease resolution, and in the absence of parasite (110, 111). In human disease, we know that *Leishmania* often persists in chronic infection and is held at bay due to the active immune response controlling further replication and expansion of *Leishmania in vivo*. This is most clearly seen in cases of immunosuppression via chemical means or naturally due to HIV infection. In active CL disease, there is often long-lived immunity following disease resolution, and this immunity is most likely related to maintenance of *Leishmania* within the host in the form of a cryptic infection (109). Thus, for any effective vaccine against *Leishmania*, the ability to induce a long-lived protective response in the absence of live persistent parasites is a key hurdle to be solved.

Concluding remarks

Overall it has been clear during the study of human and animal leishmaniasis that the immune response not only plays a key role in control of the parasite, but also in the development of pathology. While this review has focused on ATL and only briefly referred to studies on leishmaniasis in the Old World, there are many parallels between CL due to *L. major* infection. Moreover, visceral leishmaniasis in the Americas also has similar immunoregulatory aspects with KA. Thus, through continued studies designed to understand the immunological aspects of leishmaniasis, these findings will continue to aid in the development and discovery of novel therapies, vaccines, and diagnostics.

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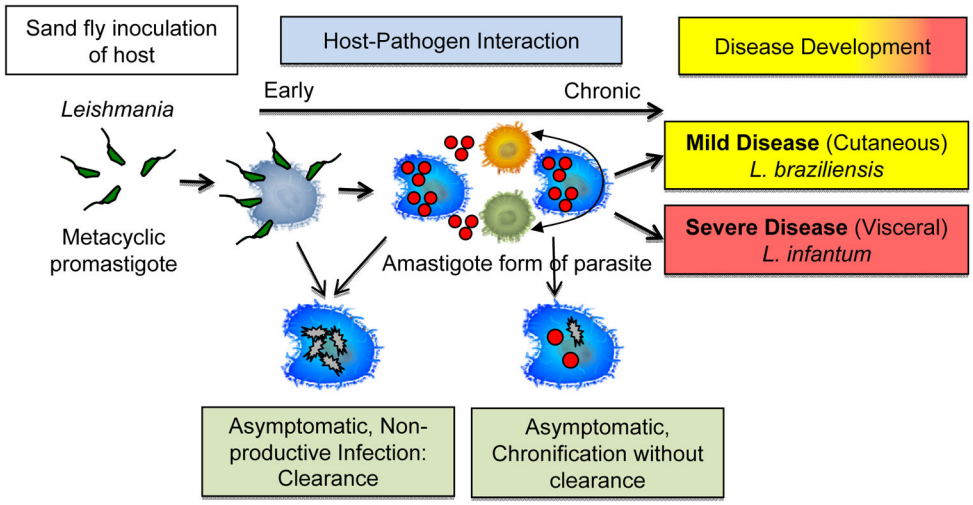

Figure 1. Parasite species and host responses influence disease outcome

Leishmania infects macrophages early during infection: 1) infection via the introduction of infective metacyclic promastigotes via an insect bite, 2) initial infection of host macrophages, 3) conversion to intracellular replicating form of the parasite (amastigote), and 4) progression to a complex host-parasite interaction culminating in diseases with distinct clinical forms.

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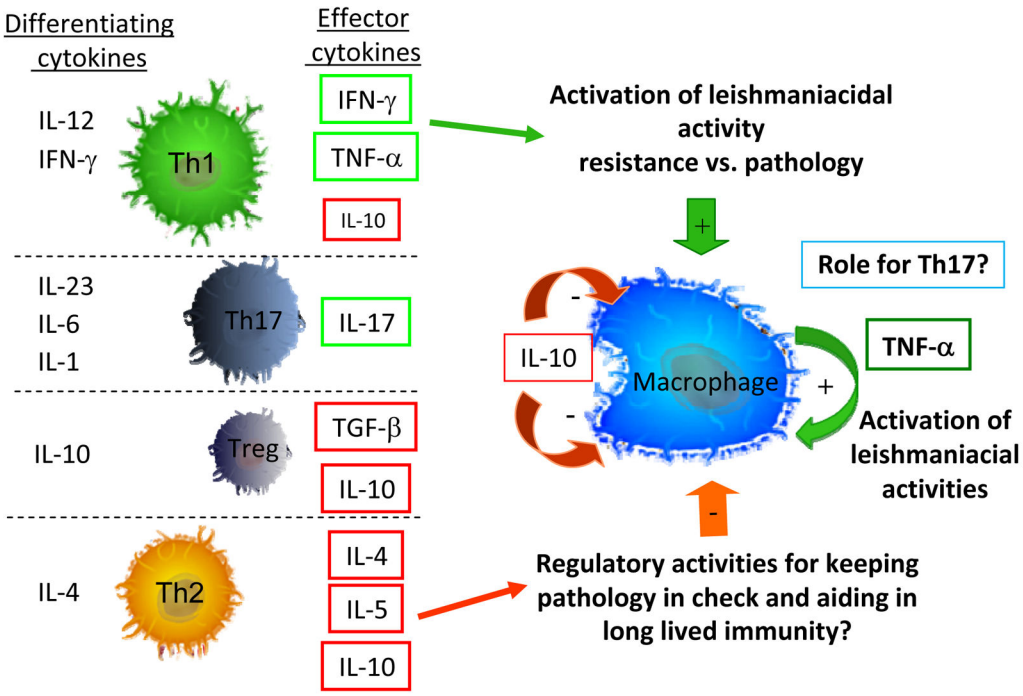

Figure 2. T cell subsets and macrophage interactions in cellular responses to *Leishmania*

Effective cellular responses to combat Leishmania depend on the formation of CD4+ T cell subsets that are capable of activating leishmaniacidal responses by host macrophages and monocytes. The differentiation of CD4+ T cell subsets, Th1, Th2, Th3, Treg and Th17 all depend greatly on the cytokine microenvironment during the initial activation of naïve CD4+ T cells. Depending on the balance of these cytokines, co-stimulatory molecules, host genetics and antigenic stimuli, a given T cell will differentiate toward one of the Th subsets and produce the effector cytokines indicated in the figure. These cytokines in turn will act on host macrophages and monocytes to prime them for effective or ineffective control of *Leishmania* and subsequent control or not of immunopathology as well.

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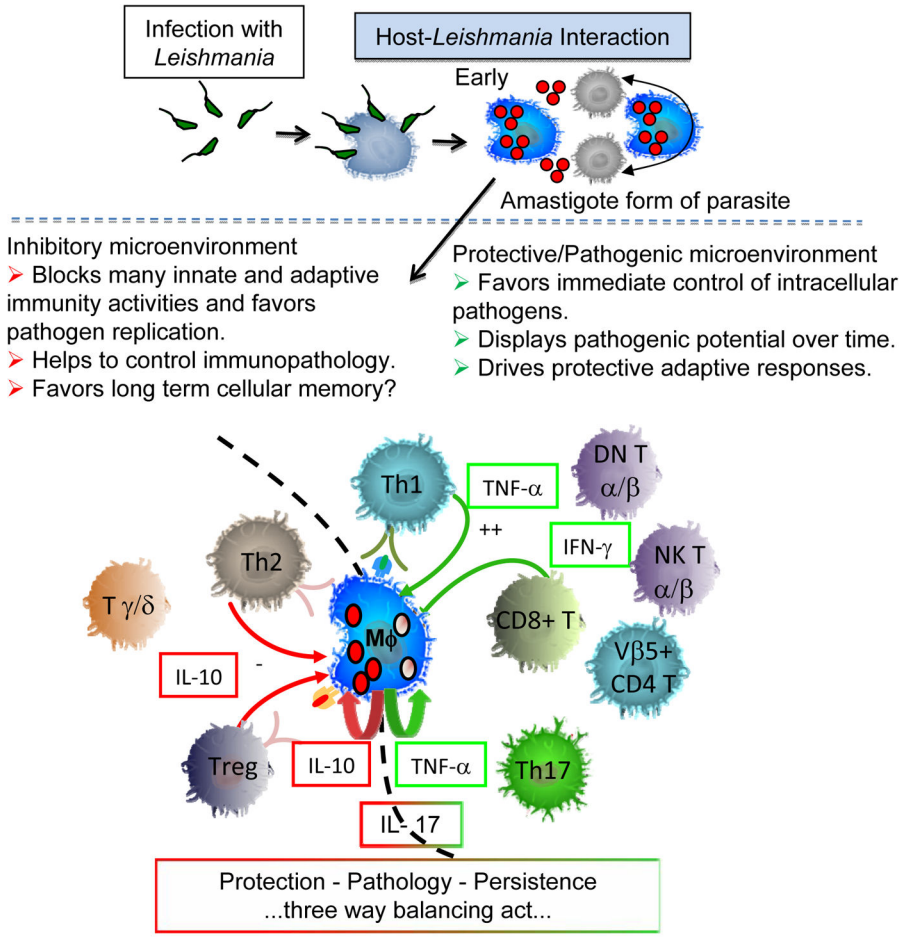

Figure 3. Protection - Pathology – Persistence, three way balancing act

After the initial introduction of the pathogen within the host, the infective form of the parasite quickly parasitizes host macrophages and converts to the amastigote, intracellular replicating form of the parasite. This initial interaction between the host and parasite is paramount for establishing the infection and directing the subsequent adaptive immune response. Both parasite and host factors interact to culminate in a beneficial or detrimental host-parasite interaction, which is dependent on multiple factors. However, it is clear that the macrophage itself will be more effective at killing *Leishmania* under a cytokine environment rich in IFN-gamma and TNF-alpha, and that IL-10 can act to reduce macrophage production of TNF-alpha and parasite killing.

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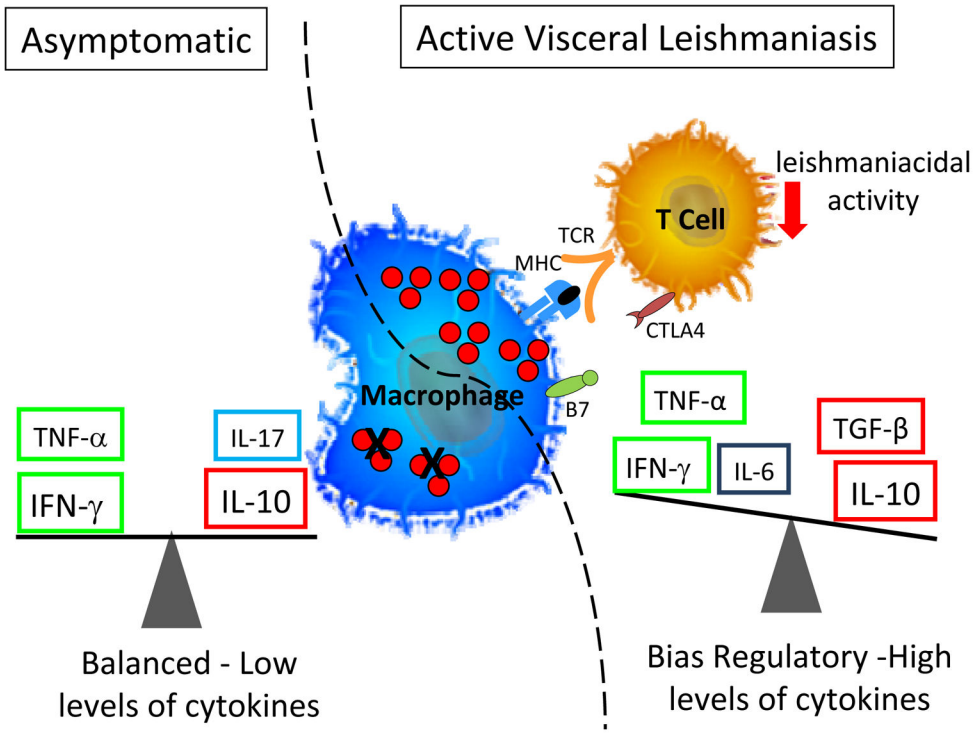

Figure 4. Representation of the two clinical outcomes following infection with *L. infantum*

While an asymptomatic outcome is associated with lower levels cytokine production and a balance between the cytokines IFN-gamma and IL-10, patients with active VL are associated with higher production of both inflammatory (IFN-gamma and TNF-alpha) and the down regulatory cytokine IL-10. Thus, a suppression of an effective leishmaniacidal response in VL is correlated with disease development, as well as the presence of immunomodulatory molecules such as CTLA-4 leading to ineffective control of *Leishmania*.