frontiers in
IMMUNOLOGY

REVIEW ARTICLE
published: 08 June 2012
doi: 10.3389/fimmu.2012.00145

The immunobiology of *Leishmania braziliensis* infection

Camila I. de Oliveira*[1,2]* and *Claudia I. Brodskyn*[1,2]

[1] *Centro de Pesquisas Gonçalo Moniz, FIOCRUZ, Salvador, Bahia, Brazil*

[2] *Instituto de Investigação em Imunologia, Salvador, Bahia, Brazil*

Edited by:
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Ricardo T. Gazzinelli, University of Massachusetts Medical School, USA

***Correspondence:**
Camila I. de Oliveira and Claudia I. Brodskyn, Centro de Pesquisas Gonçalo Moniz, CPqGM-FIOCRUZ, Rua Waldemar Falcão, 121 Salvador, Bahia, Brazil. e-mail: camila@bahia.fiocruz.br; brodskyn@bahia.fiocruz.br

Leishmaniases are a group of diseases caused by protozoa of the genus *Leishmania* that affect millions of people worldwide. These diseases are caused by distinct *Leishmania* species, of which *L. braziliensis*, a New World representative of the *Leishmania* genus, has been the least studied. Although leishmaniasis caused by *L. braziliensis* induces a range of clinical manifestations ranging from mild localized lesions to severe mucosal involvement, few studies have focused on elucidating the immune mechanisms behind this pathology. In this review, we focus on the immunobiology of *L. braziliensis* infection, emphasizing the innate and adaptive immune responses and taking into consideration both studies performed in endemic areas and experimental models of infection. Additionally, we address recent findings regarding the role of sand fly saliva in disease immunopathogenesis and vaccine development.

Keywords: *Leishmania braziliensis*, innate immunity, human adaptive immunology, animal models

INFECTION WITH NEW WORLD *LEISHMANIA* SPECIES: CLINICAL ASPECTS

Rebelo et al. (2010) proposed the term tegumentary (skin or mucosal) leishmaniasis (TL) to the French Society of Dermatology in 1925, the first contribution of Brazilian researchers to the study of this disease. TL is a major health problem in Brazil, where the main *Leishmania* species associated with this disease are *Leishmania (Viannia) braziliensis*, *L. (V.) naiffi*, *L. (V.) shawi*, *L. (V.) lainsoni*, and *L. (Leishmania) amazonensis*. Of these, *L. braziliensis* is the predominant species in the regions of Brazil where TL is endemic, and it occurs in areas of both ancient and recent colonization with a low prevalence in the Amazonas state (Jones et al., 1987). *L. braziliensis* transmission is associated with the presence of domestic animals, which are implicated as potential reservoirs. It is transmitted by several different sand fly species, including *Lutzomyia intermedia*, *Lu. whitmani*, and *Lu. wellcomei* (Miranda et al., 2002; Rebelo et al., 2010). *L. amazonensis* has been identified in different areas of Brazil and induces cutaneous ulcers, including diffuse cutaneous leishmaniasis. The main reservoirs for *L. amazonensis* are rodents and marsupials, and the main vector species associated with its transmission are *Lu. flaviscutellata* and *Lu. olmeca*. Host–parasite interactions can lead to a series of events culminating in clinical manifestations; the clinical forms of TL vary due to this complexity. In Brazil, TL can present as a single lesion (LCL, localized cutaneous leishmaniasis) that can be unapparent or discrete and can spontaneously heal. Multiple ulcerations may be present, compromising the mucosal areas (ML, mucosal leishmaniasis). ML is particularly important in South America and is caused primarily by *L. braziliensis*, although *L. amazonensis* has also been implicated (Costa et al., 1986). ML is characterized by latency and chronicity. Parasitological diagnosis is difficult, and a significant number of cases do not respond to treatment. Generally, 2–5% of TL cases in which the primary infection heals subsequently develop ML (Marsden, 1990). *Leishmania* persistence following clinical treatment may be responsible for recurrence of the disease (Schubach et al., 1998). LCL and ML represent responsive facets of the disease, with immune responses that are readily detected.

Diffuse cutaneous leishmaniasis, caused by *L. amazonensis*, is a rare but severe manifestation of the disease that develops in anergic patients and is characterized by a defective cellular immune response to *Leishmania* antigens (Convit et al., 1962). In this case, a single ulcer slowly evolves, developing plaques or multiple non-ulcerated nodules. Diffuse cutaneous leishmaniasis responds poorly to treatment, and *Leishmania* skin tests are negative.

In this review, we will focus on recent advances in understanding the complexity of TL caused by *L. braziliensis*, focusing on both experimental models of infection and pathogenesis in the human host. We will also discuss certain aspects of infection with *L. amazonensis*, given its sole association with diffuse cutaneous leishmaniasis and the singular characteristics of this disease. We aim to provide a current picture of the complex host–parasite interactions involved in leishmaniasis while also taking into account the role of the vector's saliva, an area of intense research in the past several years.

It is important to understand recent findings regarding intrinsic differences within the *Leishmania* species when addressing *L. braziliensis* pathogenesis. These differences came to light following vaccination attempts using the *L. braziliensis* homologs of the receptor for activated C kinase (LACK), thiol-specific antioxidant (TSA), *Leishmania* elongation and initiation factor (LeIF1), and *L. major* stress-inducible protein 1 (LmSTI1; Salay et al., 2007). All four open reading frames have a high degree of homology at the

amino acid level with previously described *L. major* genes. However, immunization failed to significantly reduce lesions following challenge with *L. braziliensis*.

A comparison between the genomes of *L. (L.) major*, *L. (L.) infantum chagasi*, and *L. braziliensis* showed elevated gene synteny, expected for species belonging to the same genus, with only 200 genes differentially distributed between the three species. Moreover, few genes are species-specific, and only 47 genes are specific to *L. braziliensis*. However, the greatest surprise was that genes related to RNAi machinery have only been found in *L. braziliensis* (Peacock et al., 2007). Mobile elements such as retrotransposons were also identified in *L. braziliensis* but are absent in *L. major* and *L. infantum*, both of which are RNAi-deficient (Peacock et al., 2007) Importantly, the RNAi machinery in *L. braziliensis* is functional (Lye et al., 2010) enabling the down-regulation of reporter and endogenous genes. The RNAi pathway in *L. braziliensis* could potentially help protect against mutations caused by mobile elements (Shi et al., 2004). Double-strand (ds) RNA viruses (LRVs) can infect *L. braziliensis* (Tarr et al., 1988; Patterson, 1993), and the RNAi response may help to protect against such infection (Anderson et al., 2007). However, infection with LRV increased parasite survival and pathogenesis in *L. guyanensis* (Ives et al., 2011), raising the possibility that, in RNAi-competent *Leishmania*, LRV infection may actually down-modulate RNAi activity.

INNATE IMMUNITY IN LEISHMANIASIS TRIGGERED BY *L. BRAZILIENSIS*

BALB/c mice infected in the ear dermis with *L. braziliensis* develop cutaneous lesions at the site of inoculation (de Moura et al., 2005), and histological analysis of ear sections demonstrated constant recruitment of neutrophils to the inoculation site. Interestingly, neutrophil depletion during *L. braziliensis* infection increased parasite load, whereas BALB/c mice co-inoculated with both parasites and live neutrophils displayed lower parasite loads at the site of infection and in the draining lymph nodes (Novais et al., 2009). *In vitro*, co-cultures of live neutrophils and *L. braziliensis*-infected macrophages led to a decrease in parasite load, and elimination of *L. braziliensis* elimination was associated with TNF-α and superoxide production (Novais et al., 2009). In experiments using *L. amazonensis*, phagocytosis of apoptotic human neutrophils by *L. amazonensis*-infected macrophages led to an increase in parasite burden in a TGF-β and PGE_2-dependent manner. Conversely, uptake of necrotic neutrophils by infected macrophages led to killing of *L. amazonensis*. Leishmanicidal activity was dependent on TNF-α and neutrophil elastase and was also associated with superoxide production (Afonso et al., 2008). Another function attributed to the neutrophils is the production of neutrophil extracellular traps (NETs) which are composed of filamentous genomic DNA containing antimicrobial peptides. *L. amazonensis* parasites are susceptible to killing by humans NETs, and LPG isolated from these parasites triggered NET release (Guimaraes-Costa et al., 2009).

L. braziliensis induced the production of CXCL-10 and IL-10 by human peripheral blood mononuclear cells (PBMCs) and macrophages, but the enhanced expression of CXCL10 and its receptor, CXCR3, was predominantly detected in $CD14^+$ monocytes. Interestingly, sera from TL patients, and especially those from ML patients, have significantly higher levels of CXCL10, CCL4, and soluble TNF receptor II (sTNFRII) than sera from control individuals. These multiple inflammatory mediators produced by the host may contribute to disease severity by increasing cellular recruitment (Vargas-Inchaustegui et al., 2010). However, IL-10 production is important in controlling the exacerbated inflammatory response characteristic of TL. Antonelli et al. (2004) showed a strong positive correlation between IL-10 and TNF-α-producing monocytes in PBMC cultures from LCL patients stimulated with soluble *Leishmania* antigen (SLA), suggesting that an intrinsic macrophage auto-regulation mechanism appears to be active in LCL patients.

Interferon-β increases the parasite load in infected human macrophages following infection with New World parasites (*L. braziliensis* and *L. amazonensis*) in a manner that is independent of endogenous and exogenous NO (Khouri et al., 2009). In parallel, IFN-β significantly reduces superoxide release by both *Leishmania*-infected and uninfected human macrophages. This reduction was accompanied by a significant increase in superoxide dismutase (SOD1) protein levels. Biopsies from New World cutaneous leishmaniasis patients show pronounced SOD-1 expression levels *in situ* (Khouri et al., 2009). Importantly, these results suggest that IFN-β production in human Leishmaniasis may be deleterious, particularly in DCL cases where the parasite load is elevated. Similarly, TGF-β also plays a major role in macrophage deactivation, leading to increased parasite load in an experimental model of *L. amazonensis* infection (Barral et al., 1992). Subsequently, parasite killing following *L. amazonensis* infection has been observed *in vitro* in experiments using the superoxide-dismutase inhibitor diethyldithiocarbamate (DETC; Khouri et al., 2010). Moreover, *in vivo* treatment with DETC significantly decreased lesion size and parasite load in an experimental model of *L. braziliensis* infection.

Systemic administration of *Leishmania* parasites, including *L. braziliensis,* induces *in vivo* DC maturation characterized by DC migration to T cell areas and costimulatory molecule upregulation (Antonelli et al., 2004). DCs co-cultured with *L. braziliensis* up-regulate DC activation markers and produce IL-12 and TNF-α. However, up-regulation of activation markers and IL-12 production was primarily confined to bystander (uninfected) DCs (Carvalho et al., 2008). The authors of this study proposed that bystander DCs in *L. braziliensis* infection lead to T cell activation, while infected DCs contribute to parasite control through enhanced TNF-α production. Indeed, experimentally infected $TNF\text{-}\alpha^{-/-}$ mice developed non-healing skin lesions (Rocha et al., 2007). Likewise, axenic *L. braziliensis* amastigotes successfully stimulated DCs to produce IL-12p40, inducing an activated phenotype (Vargas-Inchaustegui et al., 2008). DCs infected with *L. braziliensis* show increased phosphorylation of STAT molecules and ISG15 expression (IFN-stimulated gene 15). Accordingly, *in vivo* infection with *L. braziliensis* led to a self-healing phenotype characterized by increased numbers of IFN-γ- and IL-17-secreting $CD4^+$ T cells. DCs from $MyD88^{-/-}$ mice exhibited less activation and decreased production of IL-12 during experimental *L. braziliensis* infection, suggesting a role for TLR involvement (Vargas-Inchaustegui et al., 2009). Furthermore, $MyD88^{-/-}$ mice developed larger lesions than control mice. However, a lack of

TLR2 resulted in enhanced DC activation, increased IL-12 production and successful priming of naïve $CD4^+$ T cells. Fully understanding the role of TLRs in *L. braziliensis* infection will require further research.

ADAPTIVE IMMUNITY IN HUMAN LEISHMANIASIS

In general, patients with LCL and ML have a strong type 1 immune response to *Leishmania* antigen, with high production of IFN-γ and TNF-α and decreased efficacy of IL-10 in down-modulating IFN-γ production (Follador et al., 2002). With disease progression, ML patients tend to develop stronger intradermal skin test reactions, and their lymphocytes exhibit stronger proliferative responses and IFN-γ production than cells from LCL patients. However, antigen-stimulated PBMCs from 50% of subjects who developed the disease within the previous 60 days exhibit low or absent IFN-γ levels. This response can be restored by either IL-12 or anti-IL-10 monoclonal antibodies (Ribeiro-de-Jesus et al., 1998). Later in the disease course, both LCL and MCL patients exhibited high levels of IFN-γ and TNF-α, but TNF-α levels decreased following treatment. IFN-γ and TNF-α seem to be involved in both controlling parasite multiplication during the early phases of *Leishmania* infection and mediating the tissue damage observed in TL (Ribeiro-de-Jesus et al., 1998). In a recent study, Oliveira et al. (2011) observed a positive correlation between ulcer size at the time of the first evaluation, time to recovery, and TNF-α levels, supporting the use of TNF-α inhibitors combined with standard therapy to improve recovery time in LCL patients with severe lesions. In fact, pentoxifylline has been successfully used to decrease recovery time in ML patients, even in those that were refractory to conventional treatment (Lessa et al., 2001; Bafica et al., 2003).

Infection with *L. braziliensis* has been associated with lymphadenopathy in the absence of tegumentary lesions (skin or mucosal; Barral et al., 1992, 1995a). Lymphadenopathy can precede the appearance of skin ulcers and must be differentiated from the satellite lymph node enlargement associated with lesion establishment. Cells obtained from lymph nodes from LCL patients without ulcerations (early phase) exhibited a higher proportion of neutrophils, eosinophils, and $CD8^+$ T cells. In contrast, $CD19^+$ B cells and plasma cells were more frequently observed in patients showing lymphadenopathy with ulcerations (late phase; Bomfim et al., 2007). IL-10 transcription was significantly higher in late-phase disease, suggesting an important role for this cytokine in limiting tissue damage. IFN-γ and IL-4 levels were similar in both groups of patients, reinforcing the concept of a mixed Th1–Th2 profile during disease. These results highlight the role of $CD8^+$ T cells in the early phase of CL, as shown by Pompeu et al. (2001) using a model of *in vitro* priming. Additionally, there is a temporal relationship between ulcer development and the increased numbers of B cells. Cells from patients exhibiting ulcers typical of LCL-produced IFN-γ and TNF-α upon stimulation with SLA or LACK, which originated from multiple sources, including $CD4^+$ and $CD8^+$ T cells (Bottrel et al., 2001). A positive correlation was observed between IFN-γ or TNF-α and IL-10 production from lymphocytes. Higher frequencies of IL-10-producing parasite-specific lymphocytes are correlated with lower frequencies of TNF-α-producing monocytes, demonstrating the role of antigen-specific IL-10 production in negatively regulating monocyte activity (Antonelli et al., 2004, 2005).

Studies of different Vβ T cells in LCL patients have revealed an association between TCR Vβ 12 expressions, T cell activation and IFN-γ production after *in vitro* priming with *Leishmania* (Clarencio et al., 2006). More recently, Keesen et al. (2011) measured the expansion, activation state, and functional potential of specific T cells identified by their TCR Vβ expression. They observed an increase in CD4 Vβ 5.2- and Vβ 24-positive T cells in LCL patients compared to controls, a profile suggesting previous activation of the CD4 Vα 5.2-, 11-, and 24-positive T cells characterized by increased expression of CD45RO, HLA-DR, IFN-γ, TNF-α, and IL-10 compared to the other Vβ-expressing subpopulations and a positive correlation between higher frequencies of CD4 Vβ 5.2 T cells and lesion size. The identification of active subpopulations in this form of the disease could allow for the identification of the immunodominant *Leishmania* antigens responsible for triggering an efficient host response against the parasite and could also allow for identification of the cell populations involved in disease pathology.

In *L. braziliensis* endemic areas, approximately 10% of the individuals have a positive delayed-type hypersensitivity (DTH) skin test to *Leishmania* antigen but have neither a previous history of LCL nor a typical LCL scar. These individuals are categorized as having a subclinical (SC) *L. braziliensis* infection (Follador et al., 2002). Individuals with SC *L. braziliensis* infection produce significantly lower levels of IFN-γ and TNF-α than patients with active LCL. However, IL-10 levels are higher in these individuals than in LCL patients (Bittar et al., 2007). Recently, Novoa et al. (2011) reported stronger Th1 responses in LCL patients than in SC individuals. This finding seems be unaffected by IL-10, as levels of this cytokine at both the protein and mRNA levels were very similar in both groups. IL-27 is a cytokine that both initiates a Th1 response and regulates inflammation (Trinchieri et al., 2003; Yoshimura et al., 2006). IL-27 mRNA levels were higher in cells from LCL patients than in those from SC patients following stimulation with *L. braziliensis*. The mechanisms by which SC individuals control parasite growth are unknown. Because the adaptive immune responses in these individuals is less prominent, we can speculate that parasite control may be dependent on innate immune responses, with the participation of neutrophils (Novais et al., 2009), macrophages, and NK cells of particular importance. Interestingly, PBMC IL-17 production is slightly higher in SC patients than in LCL patients, suggesting a possible protective role for this cytokine. However, IL-17 could be exerting different functions based on the phase of disease. Our group recently illustrated the involvement of IL-17 and IL-17-inducing cytokines in biopsy specimens from ML patients. IL-17 was expressed by $CD4^+$, $CD8^+$, and $CD14^+$ cells, and numerous $IL\text{-}17^+$ cells co-expressed CCR6. We also observed the presence of neutrophils in necrotic and perinecrotic areas; these neutrophils stained positive for neutrophil elastase, myeloperoxidase, and MMP-9, indicating that IL-17 could be involved in ML pathogenesis (Boaventura et al., 2010). In fact, *in vitro* infection of human macrophages with *L. braziliensis* increased the secretion and activation of MMP-9, and macrophages from cured individuals with previous histories of ML exhibited

increased MMP-9 activity than those from cured LCL patients (Maretti-Mira et al., 2011).

Mucosal leishmaniasis patients display an exacerbated and unregulated immune response. They have a higher frequency of activated T cells than patients with LCL, as measured by different activation markers. While LCL patients displayed a positive correlation between IL-10 and TNF-α-producing monocytes, ML patients did not. This lack of correlation between IL-10-producing and TNF-α producing monocytes in ML patients could lead to a poorly controlled inflammatory response *in vivo*, and cytokine networks may be involved in the development of immunopathology in ML patients (Gaze et al., 2006). Additionally, IL-10 receptor expression was lower in ML lesions than in LCL lesions (Faria et al., 2005).

ROLE OF CD8$^+$ T CELLS

The role of cytotoxicity in host defense and tissue damage during human LCL is not yet well understood. Machado et al. (2002) observed the presence of NK cells, CD8$^+$ and CD45RO+ T cells, and strong expression of TIA-1, a molecule associated with cytotoxicity, in the dermal cell infiltrates of lesions from LCL patients. The presence of these cytolytic cells in LCL lesions suggests active participation of NK and CD8$^+$ T cells in the pathogenesis of this disease. These cells may play a role in both parasite killing and ulcer development (Machado et al., 2002). More recently, Faria et al. (2009) characterized the immunological kinetics associated with LCL progression, comparing the cellular composition and cytokine and granzyme expression in lesions of patients with early-stage (E-LCL) and late-stage LCL (L-LCL). Histopathological analysis showed that lesions from L-LCL patients displayed more exuberant inflammatory infiltration than those from E-LCL patients. Although E-LCL and L-LCL lesions were predominantly mononuclear, lesions from E-LCL patients presented higher neutrophil and eosinophil counts than those from L-LCL patients. CD8$^+$ T cells from L-LCL lesions expressed significantly higher levels of granzyme A than those from E-LCL lesions. Interestingly, granzyme A expression was positively correlated with the intensity of the inflammatory infiltrate in L-LCL patients but not in E-LCL patients. These results suggest that the recruitment of CD8$^+$ and granzyme A$^+$ T cells is involved in lesion progression in human LCL. Our group found similar results, showing that lesions from CL patients presented higher frequencies of CD8$^+$ T cells displaying CLA (cutaneous lymphocytes antigen); these cells are mainly cytolytic, with strong expression of CD107 and granzyme B. They also produce IFN-γ and IL-10, but in lower frequencies than CD4$^+$ T cells (Silva et al., submitted manuscript). Mendes-Aguiar Cde et al. (2009) also observed that the CLA receptor could direct *Leishmania*-specific CD8$^+$ T lymphocytes toward inflamed skin lesions, suggesting that *Leishmania* antigens could modulate the molecules responsible for skin lesions, affecting the cell composition of the inflammatory infiltrate in Leishmaniasis. CD8$^+$ T cells seem to play distinct roles in different phases of the disease. Using an *in vitro* priming assay (IVP), we observed that CD8$^+$ T cells are the first cells to be activated by *Leishmania* promastigotes and to produce IFN-γ, which is partially responsible for directing the differentiation of Th1 cells (Pompeu et al., 2001). However, CD8$^+$ T cells and NK cells also contribute to the tissue destruction observed in ML patients in late stages of disease (Brodskyn et al., 1997).

IMMUNOSUPPRESSION IN LEISHMANIASIS

In spite of a robust immune response, a small number of parasites persist following the resolution of leishmaniasis (Mendonca et al., 2004; Figueroa et al., 2009; Martins et al., 2010). In mice infected with *L. braziliensis* in the ear dermis, parasites also persist in draining lymph nodes, despite lesion resolution and parasite clearance from the infection site (de Moura et al., 2005). In mice, IL-10 blockade following infection with low doses leads to a sterile cure of the disease (Belkaid et al., 2001). In Leishmaniasis, this cytokine can be produced by several different cell sources, including Treg cells (Belkaid et al., 2002), Th1 cells (Stager et al., 2006; Anderson et al., 2007; Nylen et al., 2007); CD8$^+$ T cells (Belkaid et al., 2002), B cells (Ronet et al., 2010), NK cells (Maroof et al., 2008), regulatory DCs (Svensson et al., 2004), macrophages (Miles et al., 2005), and neutrophils (McFarlane et al., 2008). In patients, functional Treg cells could be found in the skin lesions of patients with LCL. These cells expressed phenotypic markers of Treg cells, including CD25, CTLA-4, Foxp3, and GITR (glucocorticoid-induced tumor necrosis factor receptor), and were able to produce large amounts of IL-10 and TGF-β. CD4$^+$CD25$^+$ T cells derived from the lesions of patients with LCL suppressed the PHA-induced proliferative T cell responses of allogeneic PBMCs from healthy controls (Campanelli et al., 2006). These findings suggest that functional Treg cells accumulate at sites of *Leishmania* infection in humans and possibly contribute to the local control of effector T cell functions.

Another important immunosuppressive cytokine involved is TGF-β. This cytokine has different effects on cells in the immune system, including down-regulation of certain macrophage functions. As commented earlier, this cytokine is produced by macrophages present in the lesions of mice infected with *L. amazonensis* (Barral-Netto et al., 1992). Human macrophages produce active TGF-β after infection with *L. amazonensis*, *L. chagasi*, and *L. braziliensis*. The addition of this cytokine to cultures of *L. braziliensis*-infected macrophages led to an increase in parasite numbers compared to untreated cultures. Fibroblasts in the dermis could be immunostained for TGF-β, as could inflammatory cells from the biopsies of human lesions on occasion – mainly from patients with early cutaneous leishmaniasis (less than 2 months following ulcer development) and in cases of active mucosal leishmaniasis (Barral et al., 1995b). These results suggest that this cytokine could be important for the establishment of infection and as a feedback mechanism following the development of an acute inflammatory response.

EXPERIMENTAL INFECTION WITH *L. BRAZILIENSIS*: INSIGHTS INTO HUMAN PATHOGENESIS?

Initial studies conducted with inbred mice infected with *L. braziliensis* revealed a broad range of responses. AKR/J and CBA/J mice showed only a mild and transient swelling of the nose. SWR/J, C57L/J, A/J, A/HeJ, and DBA/1J mice showed initial nodules, which eventually healed. In contrast, BALB/cJ mice were considered susceptible based on progressive dermal lesions (Childs et al., 1984). However, analysis of the immune response has

shown that *L. braziliensis*-infected BALB/c mice produce less IL-4 than *L. major*-infected mice, and treating *L. braziliensis*-infected BALB/c mice with anti-IFN-γ significantly enhanced lesion size and prevented mice from resolving the infection (DeKrey et al., 1998). These authors suggest that an IFN-γ-dependent mechanism is responsible for killing *L. braziliensis* in BALB/c mice and that the weak infectivity of *L. braziliensis* in this mouse strain may be due to the inability of the parasite to elicit a strong and sustained IL-4 production. In a recent study, mice inoculated with an antimony-resistant *L. braziliensis* strain displayed an increased IL-4 response and elevated Arginase I expression (Costa et al., 2011). Treatment with an anti-IL-4 monoclonal antibody resulted in decreased lesion thickness and parasite load. Therefore, the capacity of *L. braziliensis* isolates to induce a Th2-type response, characterized by presence of IL-4, contributes to the virulence and severity of disease.

Using the footpad model of infection, we showed that mice infected with a *L. braziliensis* strain from Ceará (H3227) developed detectable lesions, whereas mice infected with a different strain (BA788), isolated in Bahia, did not (Indiani de Oliveira et al., 2004). Early after parasite inoculation, lymph node cells from BA788-parasitized mice produced higher levels of IFN-γ and had higher numbers of NK cells than H3227-infected mice. Importantly, the *L. braziliensis* strain from Ceará (H3227) is genotypically different from the *L. braziliensis* strain from Bahia (BA788). Therefore, variation in the pathogenicity of these different *L. braziliensis* strains correlated with their genetic diversity. Interestingly, in Ceará state, where the H3227 *L. braziliensis* isolate was obtained, prominent lymphadenopathy can precede skin lesion appearance (Sousa Ade et al., 1995), suggesting that important correlates can be drawn from the mouse model. In a subsequent study using the same two strains, the H3227 *L. braziliensis* strain induced significantly stronger cellular recruitment than the BA788 strain, which correlated with higher expression of CCL2, CCL3, and CXCL1 (Teixeira et al., 2005). In contrast, *L. braziliensis* BA788 significantly up-regulated CXCL10 expression, which correlated with earlier IFN-γ production and with a higher number of NK cells present at the infection site (Indiani de Oliveira et al., 2004).

BALB/c mice infected with a *Leishmania* strain isolated from a ML patient developed a rapidly progressing and widely metastatic disease resembling diffuse cutaneous leishmaniasis (Barral et al., 1983). Although C57BL/6 mice initially contained parasite multiplication and appeared clinically cured, subsequent disease developed that was characterized by distinctive ulcerative metastases and destruction of the nasal region, similar to what is observed in ML. Disease development in these mouse strains was associated with a decrease in cell-mediated immunity, as monitored by delayed type hypersensitivity and lymphoproliferative responses. Footpad injection of metacyclic *L. braziliensis* into C57BL/6 mice confirmed the initial findings regarding disease outcome in this mouse strain, as the mice control infection and parasite multiplication in the draining lymph nodes (Maioli et al., 2004). This phenotype was associated with increased levels of IFN-γ and TNF-α and a superior lymphoproliferative response. Importantly, in the same study, the authors demonstrated that *L. amazonensis* infection leads to chronic lesion development with elevated parasite numbers and a decreased cellular response, highlighting the differences in immune regulation induced by the distinct New World *Leishmania* species.

As has been thoroughly described in the literature, mice from BALB strains are highly susceptible to *L. major* infection, and this susceptibility is linked to a predominant Th2 response characterized by the presence of IL-4 (reviewed in Belkaid et al., 2002). In a comparative study, BALB/c mice infected subcutaneously with *L. braziliensis* developed small, nodular lesions that self-healed, in contrast to *L. major*-infected BALB/c mice, which displayed progressive ulcers (Rocha et al., 2007). This phenotype was confirmed by intradermal infection with *L. braziliensis*, after which mice develop ulcerated lesions (similar to the lesions that develop upon natural infection) that heal spontaneously (de Moura et al., 2005). In this model, a mixed Th1/Th2 immune response was observed that was characterized by the presence of IFN-γ, IL-4, and IL-10-secreting cells, which is distinct from the Th2-polarized response observed following *L. major* infection. Interestingly, parasites are cleared from the infection site following lesion healing but persist within draining lymph nodes, suggesting that immunoregulatory mechanisms allow for parasite survival. Indeed, the presence of $CD4^+CD25^+$ T cells expressing regulatory markers such as Foxp3, GITR, and CD103 has been described following *L. braziliensis* infection (Costa et al., 2011; Falcão et al., 2012).

A similar phenotype was also observed in C57BL/6 mice. The "resistance" observed in *L. braziliensis*-infected mice of this strain was associated with significantly lower IL-4 and IL-13 production in parallel with increased presence of IFN-γ and iNOS. Previous work has shown that IFN-$\gamma^{-/-}$ mice infected with *L. braziliensis* develop uncontrolled lesions (DeKrey et al., 1998), while IL-12p40$^{-/-}$ mice, which lack both IL-12 and IL-23, develop chronic lesions (de Souza-Neto et al., 2004). As lymphocytes from the latter produce decreased levels of IFN-γ, this further implicates IFN-γ in the control of *L. braziliensis* infection. These results were confirmed by infecting IL-12p35$^{-/-}$ mice, which lack IL-12 only. These mice display uncontrolled lesions, as do IL-12p35p40$^{-/-}$ mice (Rocha et al., 2007). A similar phenotype was observed following infection of STAT4$^{-/-}$ mice, implicating IL-12 in the immune response to *L. braziliensis*. Lastly, iNOs$^{-/-}$ mice develop progressive non-healing lesions and have an increased parasite load within the draining lymph nodes (Rocha et al., 2007). The lesions self heal in mice lacking gp91phox, indicating that only iNOS is essential for controlling *L. braziliensis* infection.

NEW WORLD LEISHMANIASIS AND THE ROLE OF SAND FLY SALIVA

Several attempts have been made to reproduce the biology of natural transmission, taking into account parasite load (sand flies inoculate low numbers of parasites), the presence of saliva (parasites are injected into the host's skin in conjunction with sand fly saliva) and the site of inoculation (parasites are injected by the sand fly into the dermal compartments of the skin). BALB/c mice inoculated in the ear dermis with *L. braziliensis* develop ulcerated lesions (de Moura et al., 2005), but co-inoculation of parasites plus sand fly saliva exacerbates infection

(Samuelson et al., 1991), which is associated with elevated IL-4 production (Lima and Titus, 1996). Similarly, co-inoculation with *L. amazonensis* and sand fly saliva also exacerbates infection; however, in this context, it was associated with increased IL-10 levels (Norsworthy et al., 2004). Moreover, mice injected with parasites in the presence of saliva developed lesions with heavily parasitized epithelioid macrophages, persistent neutrophils and eosinophils, and minimal fibroplasia, indicating that sand fly saliva modifies the inflammatory response during infection with *L. braziliensis*. Of note, this disease exacerbation in the presence of saliva was dependent on the sand fly species, as co-inoculation with *L. braziliensis* and *Lu. whitmani* saliva led to the development of lesions that heal spontaneously (Bezerra and Teixeira, 2001).

Pre-exposure to saliva or to bites from uninfected sand flies has been shown to result in protection against subsequent infection with New World species such as *L. amazonensis* (Thiakaki et al., 2005) and *L. chagasi* (Gomes et al., 2008). In contrast, pre-exposure to *Lu. intermedia* saliva enhanced *L. braziliensis* infection in the mouse model; additionally, disease exacerbation was correlated with the generation of a Th2 response, as evidenced by a reduction in the IFN-γ/IL-4 ratio (de Moura et al., 2007). Importantly, individuals with active LCL showed stronger humoral immune responses to *Lu. intermedia* saliva than control subjects, a finding also true for Old World LCL (Rohousova et al., 2005). These data indicate an association between disease and the immune response to *Lu. intermedia* saliva in humans. As mentioned earlier, sand fly saliva modifies the inflammatory response to infection with *L. braziliensis* (Lima and Titus, 1996). Indeed, pre-treatment of human monocytes with *Lu. intermedia* saliva followed by *L. braziliensis* infection led to a significant increase in TNF-α, IL-6, and IL-8 production (Menezes et al., 2008), indicating the capacity of *Lu. intermedia* saliva to alter the inflammatory milieu. Stimulation with *Lu. intermedia* salivary proteins markedly increased leukocyte recruitment, and mice immunized with *Lu. intermedia* saliva showed a concomitant increase in CXCL1, CCL2, CCL4, and TNF-α expression (de Moura et al., 2010). Upon stimulation with *L. braziliensis*, however, IL-10 up-regulation was observed, confirming that pre-exposure to *Lu. intermedia* is able to modify the inflammatory environment and, in doing so, favors *L. braziliensis* establishment.

Several groups have examined the possibility of vaccination using salivary antigens. Immunization with a DNA plasmid coding for the SP15 *Phlebotomus papatasi* protein was effective in providing protection against infection by *L. major* (Valenzuela et al., 2001). In parallel, we have shown that hamsters immunized with a DNA plasmid coding for LJM19, a protein present in *Lu. longipalpis* saliva, were protected against the development of visceral leishmaniasis (Gomes et al., 2008). LJM19-immunized hamsters maintained a low parasite load that correlated with high IFN-γ/TGF-β ratio and iNOS production. Importantly, a delayed-type hypersensitivity (DTH) response with high expression of IFN-γ was also detectible in the skin of LJM19-immunized animals. These studies suggested that a DTH response generated against salivary antigens such as SP15 or LJM19 might be the mechanism underlying protective anti-*Leishmania* immunity. Based on this hypothesis, we have shown that immunization with a DNA plasmid coding for LJM19, a protein present in *Lu. longipalpis* saliva, conferred protection against *L. braziliensis* (Tavares et al., 2011). These results suggest the possibility of using salivary antigens to generate protection against different species of *Leishmania*.

CONCLUDING REMARKS

Although *Leishmania* infection has been widely used to elucidate many aspects of the immune response to intracellular pathogens, we still do not fully understand the immunopathogenesis of the human disease. It is clear that LCL and ML caused by *L. braziliensis* are diseases in which immunoregulation, rather than parasite multiplication *per se*, plays a major role. Moreover, DCL caused by *L. amazonensis* is a disease that completely lacks a cellular immune response. Collectively, these major differences urge for more studies focused on human immunology. The experimental mouse intradermal infection model using *L. braziliensis* recapitulates many aspects of the human infection; as such, it will prove a useful tool for dissecting aspects of both the innate and the adaptive immune response to this important representative of New World leishmaniasis.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 14 February 2012; accepted: 18 May 2012; published online: 08 June 2012.

Citation: de Oliveira CI and Brodskyn CI (2012) The immunobiology of Leishmania braziliensis infection. Front. Immun. ***3****:145. doi: 10.3389/fimmu.2012.00145*

This article was submitted to Frontiers in Microbial Immunology, a specialty of Frontiers in Immunology.