The Dissertation Committee for Diego A. Vargas Inchaustegui certifies that this is the approved version of the following Dissertation:

REGULATION OF INNATE IMMUNE RESPONSES TO *LEISHMANIA BRAZILIENSIS* INFECTION

Committee:

Lynn Soong, M.D., Ph.D., Supervisor

A. Clinton White, M.D.

Nigel Bourne, Ph.D.

Janice J. Endsley, Ph.D.

Kai Zhang, Ph.D.

Dean, Graduate School

REGULATION OF INNATE IMMUNE RESPONSES TO *LEISHMANIA BRAZILIENSIS* INFECTION

by

Diego Antonio Vargas Inchaustegui

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Dedication

To Alison, for always being there. Te amo.

To my parents, for always pushing me to reach higher.

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ABSTRACT

Leishmania braziliensis (*Lb*) is a protozoan parasite, and the causative agent of cutaneous (CL) and mucocutaneous (ML) leishmaniasis in South America. ML is a severe and disfiguring form of the disease, usually compromising mucosal tissues within the nose, mouth and pharynx. In humans, ML is characterized by excessive T and B cell responses to the parasite, which contribute to inflammation and tissue destruction. Since our understanding of how *Lb* parasites interact with dendritic cells (DCs) is limited, the present dissertation was aimed at studying the immune processes that take place during *Lb* infection. Our results have revealed three unique features of *Lb* infection. First, using a mouse model we showed that DCs can efficiently recognize, process and present parasite antigens to $CD4^+$ T, cells initiating the development of protective immune responses. Specifically, *Lb*-infected DCs induced the proliferation of naïve $CD4^+$ T cells and the production of IFN-γ and IL-17. Second, we demonstrated that interaction between pathogen recognition receptors and *Lb* parasites occurs both *in vitro* and *in vivo*, and that these interactions modify DC activation and infection outcome. We found that whereas the adaptor protein MyD88 was necessary for the establishment of a protective immune response against *Lb* infection, the absence of TLR2 did not increase mouse susceptibility to infection. Finally, by using a human peripheral blood mononuclear cell (PBMC) *in vitro* system for *Lb* infection, we evaluated the early innate immune events associated with infection. Our results showed that within PBMCs, monocytes were the target cell for *Lb* infection, resulting in the production of inflammatory chemokines. By comparing our human *in vitro* studies to the chemokine profile observed in CL and ML patients, we revealed a potential association between the magnitude of the inflammatory response and disease severity. Collectively, our studies have contributed to an advanced understanding of *Lb* pathogenesis, in particular the roles played by DCs and monocytes. Furthermore, our data has identified several molecules for future studies to better understand the host- and pathogen-associated mechanisms of pathology and disease control.

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LIST OF ABREVIATIONS

Lb	*Leishmania* (*Viannia*) *braziliensis*
La	*Leishmania amazonensis*
Pm	Promastigotes
Am	Amastigotes
CL	Cutaneous leishmaniasis
ML	Mucocutaneous leishmaniasis
DCL	Diffuse cutaneous leishmaniasis
VL	Visceral leishmaniasis
DC	Dendritic cell
LN	Lymph node
BMDC	Bone marrow-derived dendritic cell
PBMC	Peripheral blood mononuclear cell
LPG	Lipophosphoglycan
MΦ	Macrophage
B6	C57BL/6J mice
B/c	BALB/c mice
PV	Parasitophorous vacuole
IMDM	Iscove's modified Dulbecco's Media
GM-CSF	Granulocyte macrophage colony-stimulating factor
FACS	Fluorescent-activated cell sorting
CFSE	Carboxyfluorescein succinimidyl ester
APC	Antigen-presenting cell
JAK	Janus Kinase
STAT	Signal transducer and activator of transcription
MAPK	Mitogen-activated protein kinase
IRF	Interferon regulatory factor
ISG15	Interferon stimulated gene 15
TLR	Toll-like receptor
MyD88	Myeloid differentiation primary response gene 88
p.i.	Post infection

CHAPTER 1: INTRODUCTION

LEISHMANIA PARASITES AND LEISHMANIASIS

Leishmaniasis is a vector-transmitted disease that is caused by the intracellular protozoan parasite *Leishmania sp*. Of the 32 species identified, about 20 are pathogenic for humans and other vertebrate mammals such as rodents and dogs. The disease is considered to be one of the top six major tropical diseases by the World Health Organization (WHO), and it is spread throughout 88 countries in the tropical and sub-tropical areas of the world. Currently, 12 million people worldwide are infected by the parasite, and approximately 350 million people are at a high risk of acquiring it, while no vaccination strategy is presently available (1-3). *Leishmania* parasites are taxonomically classified within the order Kinetoplastida and the family Trypanosomatidae (6).

The genus *Leishmania* has 2 subgenera named *Leishmania* (*L.*) and *Viannia* (*V.*). A first approach to categorize *Leishmania* parasites was done by grouping them in different complexes within these genera. Table 1.1 shows the most studied species from each of the known *Leishmania* complexes. It is important to mention that in modern literature *Leishmania* species are commonly referred to by using only the genus and

species information. For example, *Leishmania (Viannia) braziliensis* is commonly referred to as *L. braziliensis*, or simply *Lb*.

Subgenus	Complex	Members (Species)
Leishmania (L.)	*donovani*	*L. (L.) donovani, L. (L.) infantum, L. (L.) chagasi*
Leishmania (L.)	*mexicana*	*L. (L.) amazonensis, L. (L.) mexicana, L. (L.) pifanoi*
Leishmania (L.)	*tropica*	*L. (L.) tropica, L. (L.) major, L. (L.) aethiopica*
Leishmania (L.)	*hertigi*	*L. (L.) hertigi*
Viannia (V.)	*braziliensis*	*L. (V.) braziliensis, L. (V.) guyanensis, L. (V.) peruviana*

Table 1.1 Classification of *Leishmania* species in different complexes.

A second categorization strategy used for *Leishmania* parasites is based on their geographic distribution, being either New or Old World species. *Leishmania* parasites are transmitted by the bite of infected female sand flies, and different species of these flies are responsible for transmission in different geographic areas of the world. For example, Old World species such as *L. major* depend on the sand fly vector *Phlebotomus papatasi* for their transmission in areas like northern Africa and Asia (7). Similarly, the New World species *L. mexicana* is linked to the sand fly vector *Lutzomya longipalis* in the tropical areas of the American continents (8).

Historically, the first descriptions of *Leishmania* parasites date back to 1903, when the first reported cases of "Dum-dum fever" were described by India-based British army physician, Sir William B. Leishman (9). However, at that time there was no epidemiological explanation, or known causative agent for this disease. Leishman described the finding of small "oval bodies" in spleen smear preparations while conducting the post-mortem of a British soldier that had died of this disease. Concurrently, Army physician Captain Charles Donovan – also on duty in India, and who had also been looking for the cause of dum-dum fever – found the same oval bodies both in spleen and blood tissues obtained from local people that had died from what he called a "malaria-like" malignancy (10). Donovan's findings did not only corroborate Leishman's reports, but also strengthened the fact that a new species of trypanosomatid parasite had been discovered. Following these two reports, the genus *Leishmania* was created and *Leishmania donovani* was the first species reported.

THE LIFE CYCLE OF *LEISHMANIA* PARASITES

Despite their differences in geographic distribution and transmission, all *Leishmania* parasites are obligate, intracellular dimorphic organisms. The term "dimorphic" refers to the two different developmental forms that exist either extracellularly in the vector, or intracellularly in the mammalian host, which are termed promastigotes and amastigotes, respectively. *Leishmania* parasites are maintained in

nature within vertebrated mammal hosts, mainly dogs and rodents, that act as reservoirs for the disease.

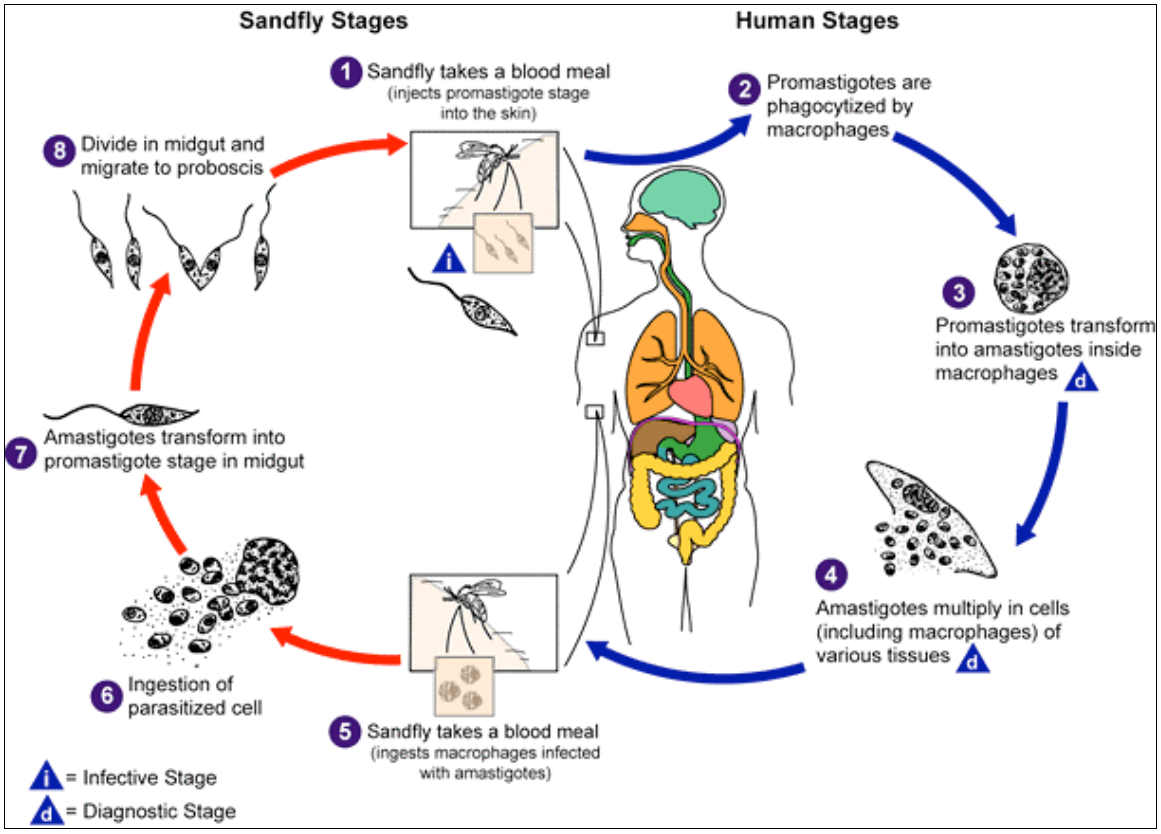

Fig. 1.1 The life cycle of *Leishmania* parasites. *Diagram modified from the Leishmaniasis Image library, Division of Parasitic Diseases, Centers for Disease Control and Prevention* (4).

The *Leishmania* life cycle begins when metacyclic promastigotes that reside in the midgut of infected female sand flies are transmitted to the host during a blood meal (Fig. 1.1, number 1). While they are feeding, sand flies also salivate on their hosts, and the inoculation of these salivary gland products helps the feeding process by causing vasodilation (11). As the current model depicts in Fig. 1.1, the parasites that are inoculated into the skin are phagocytized by MΦs, where they survive and replicate (as non-motile intracellular amastigotes) in the compartment known as the parasitophorus vacuole (PV). However, recent literature has showed that neutrophils play an important role during the initial stages of parasite infection. First, van Zandbergen *et al.* reported that *Leishmania* parasites use neutrophils as "Trojan horses" in order to silently infect MΦs, avoiding the activation of anti-microbial mechanisms in these cells (12). More recently, using dynamic intravital microscopy, it has been reported that during *in vivo* infection, neutrophils are the first cells to arrive at the bite site and to capture the invading parasites (13). However, as these experiments were followed for several hours post-infection (p.i.), the investigators found no evidence of *Leishmania*-infected neutrophil uptake by MΦs, therefore disagreeing with the "Trojan horse" hypothesis. Nevertheless, this study also proved that neutrophils were necessary for successful establishment of infection, as neutrophil-depleted mice developed smaller lesions when compared to non-depleted controls (13).

Despite the different mechanisms of entry into MΦs, it is widely accepted that MΦs are the primarily host cell for *Leishmania* survival and growth, and that efficient

MΦ activation is the only path to parasite elimination (14). Parasite survival can also occur in other immune cells such as DCs and neutrophils; however, the reduced growth capacity of the parasite in these cells limits their potential as long-term reservoirs. The *Leishmania* life cycle continues when excessive parasite growth within the PVs disrupts cell integrity, allowing amastigotes to infect neighboring cells (Fig. 1.1, number 4). The cycle is completed when un-infected sand flies take a blood meal from an infected host. In this case, the sand fly ingests infected macrophages, which upon digestion in the sand fly stomach will release the intracellular amastigotes (Fig. 1.1, number 6). The environmental change in temperature, pH and nutrients induces amastigotes to revert into the vector-fitted promastigote stage, which survives and replicates in the sand fly midgut (Fig. 1.1, number 8).

CLINICAL MANIFESTATIONS IN HUMANS

Human clinical manifestations are roughly divided into four distinct categories. The most common manifestation is cutaneous leishmaniasis (CL, Fig. 1.2A), which worldwide is endemic in more than 70 countries. The vast majority of CL cases (~90%) occur in Afghanistan, Algeria, Brazil, Pakistan, Peru, Saudi Arabia and Syria (15). CL, often referred to as localized CL, is characterized by the formation of a small area of erythema at the site of infection. In a period of time that varies from patient to patient (2 wk to 6 months), the erythema develops into a papule that progressively ulcerates and

vesiculates and forms an ulcer. Lesions vary in severity, size and self-cure rate, which are all often directly correlated to the parasite species causing the disease (1). CL lesions can often aggravate due to secondary bacterial or fungal infections, which complicates treatment and disease outcome (16). Pentavalent antimonials are the most common drugs used to treat leishmaniasis; however, upon lesion healing, patients are often left with disfiguring scars, which negatively affect their socio-economical expectations (17). The second and third categories of disease are called mucocutaneous leishmaniasis (ML, Fig. 1.2B) and diffuse cutaneous leishmaniasis (DCL, Fig. 1.2C), respectively. ML is primarily caused by *Lb* infection, and as *Lb* is the particular species under study within this dissertation, the characteristics of ML will be discussed with more detail in the next section.

Despite being considered two distinct types of clinical manifestations, both ML and DCL originate from a primary cutaneous lesion (18). DCL differs from CL in that the architecture of the initial cutaneous lesion is lost, therefore allowing the formation of "satellite lesions" (Fig. 1.2C). Most cases of DCL are associated with either *L. amazonensis* (*La*) or *L. aethiopica* infection, and are characterized by higher lesional MΦ-to-T cell ratios, unlike healing CL lesions where T cells are present at higher frequencies (19). Due to this low frequency of parasite-specific T cells, DCL patients have an absent DTH response to *Leishmania* antigens (20, 21).

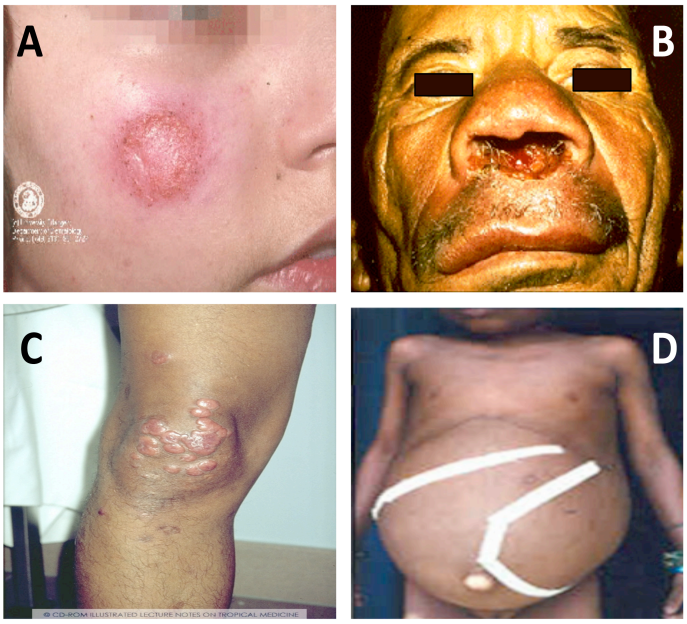

Fig. 1.2 Clinical manifestations of leishmaniasis. (A) Facial skin lesion due to CL. (B) Mucosal lesion in the nasal septum due to ML. (C) Numerous skin lesions due to DCL. (D) Expanded abdomen caused by spleen and liver enlargement due to VL. *All images courtesy of WHO/TDR.*

Systemic infection with certain *Leishmania* species leads to the fourth type of clinical manifestation called visceral leishmaniasis (VL, Fig. 1.2D). VL is both the most severe and most frequently fatal form of leishmaniasis, making it the second-largest parasitic killer in the world, only surpassed by malaria (22). The vast majority of new VL cases occur in India, Bangladesh, Nepal, Sudan and Brazil. Within the Old World, VL is caused by infection with *L. donovani* and *L. infantum*, and in the New World by *L. chagasi* (23). Most individuals exposed to VL-causing *Leishmania* parasites show no clinical signs of infection, making early kinetic studies in human populations difficult (24). However, for the 5-10% of individuals that do develop disease, symptoms include spleen and liver enlargement, as well as recurrent fever and cachexia (25). Progressive

disease leads to parasite spreading into the liver, spleen and bone marrow, where infection greatly impairs the host's immunity and becomes deadly if left untreated (26).

L. BRAZILIENSIS AND MUCOCUTANEOUS LEISHMANIASIS

Most *Lb*-infected individuals develop CL initially; however, 5-10% of patients further progress into ML in a period of time that can vary from months to several years. It is important to mention that even though *Lb* is the main causative agent of ML, reports have shown that ML can also rarely be caused by infection with *La*, *L. guyanensis* and *L. panamensis* (27, 28). ML is a severe and disfiguring form of the disease, in which lesions generally start in the nasal septum and from there they progress into the nasal cavity, the pharynx, soft palate, vocal chords and finally to the trachea (Fig. 1.3).

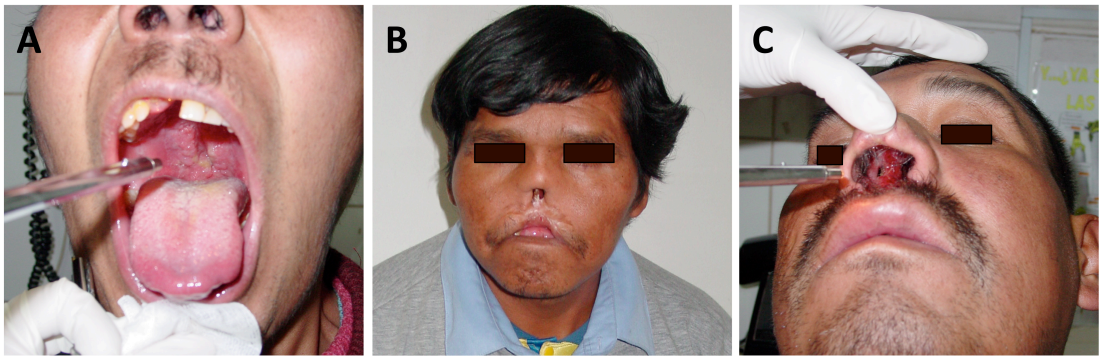

Figure 1.3 Clinical magnifications of mucocutaneous leishmaniasis. Mucosal lesions in ML can extend to nose, nasopharaynx, palate, epiglottis, larynx, vocal cords, and trachea. (A) Mucosal lesions in the palate. (B) Extensive nasal and mucosal tissue destruction caused by ML. (C) Loss of nasal septum due to ML. *All pictures courtesy of Lynn Soong, MD, PhD.*

In most cases, ML lesions become evident several years after resolution of the original CL lesion, even though some cases evolve while the CL lesion is still present. Initially, ML lesions appear as mucosal ulcerations within the hard and/or soft palate regions. Histologically, ML lesions are characterized by a sub-epithelial mononuclear inflammatory reaction mainly composed of lymphocytes, plasma cells and MΦs (29). Recently, Amato *et al.* have reported that active ML lesions (pre-treatment) have a significant higher density of MC when compared to treated ML lesions, where the density of MC decreases after healing. Furthermore, MC density was inversely correlated to IFN-γ and IL-4 expression (30). Because MC have been associated with chronic inflammatory diseases, these findings expand the current knowledge on the composition of ML lesions. Visualization of lesion tissue sections with hematoxylin-eosin staining can reveal inclusions in the cytoplasm of MΦs (amastigotes-like "oval bodies"); however, other organisms such as *Toxoplasma sp.* and *Histoplasma sp.* can cause similar cytoplasmic inclusions. Definitive histological diagnostic can be performed either by Giemsa stain or the use of *Leishmania*-specific antibodies for Immunofluorescence (31).

The immunopathogenic competence (ability of the parasite to deviate T cell immune responses) plays an important role during *Lb*-mediated development of ML. In this context, *Lb* parasites show a marked tendency to induce Th1-type immune responses, where elevated levels of IFN-γ and TNF-α in the serum are associated with necrosis of nasopharyngeal and oral mucosal tissues (32). In contrast, in *La*-mediated DCL the nodular and disseminating skin lesions are linked to a high production of IL-4, which

explains the weakened T cell immune response that favors parasite survival (33). Table 1.2 compares the main immunological characteristics of human DCL and ML disease manifestations. ML lesions are often considered to be "T cell hypersensitivity" areas, where $CD4^+$ T cells outnumber $CD8^+$ T cells (34). The abundance of $CD4^+$ T cells accounts for the high levels of IFN-γ and TNF-α observed in this extreme and disfiguring hypersensitivity form of clinical manifestation.

Human	***L. amazonensis***	***L. braziliensis***
Clinical manifestation	DCL	ML
Immunological features		
- Immunopathogenesis	Profound immune suppression T cell hyposensitivity	Excessive T & B cell responses T cell hypersensitivity
- DTH	Negative	Positive
- T_h balance	$T_h2 > T_h1$	$T_h1 > T_h2$
- Lesional T cells	$CD8^+ > CD4^+$	$CD4^+ > CD8^+$
- Cytokines	High IL-4, low IFN-γ	Low IL-4, high IFN-γ
Responsiveness to drugs	Very poor	Highly variable
DC responses (*in vitro* evidence)	Minor DC activation	Not studied

Table 1.2 Differential human immune responses to two New World species of *Leishmania*. *La and Lb* infections in humans cause initial cutaneous lesions; however, both infections can result in rare but unique disease manifestations. DCL, diffuse cutaneous leishmaniasis; ML, mucocutaneous leishmaniasis; DTH, delayed-type hypersensitivity (also known as Montenegro's skin Test).

Other key immunological features of *Lb*-mediated ML disease that have been reported in patients include: a) elevated levels of serum anti-*Lb* IgG antibodies (especially IgG1 and IgG3) (35); b) increased levels of Th1 responses (IFN-γ and TNF-α and IL-6) in stimulated peripheral blood mononuclear cells [PBMCs] (36); c) elevated

frequency of effector and memory $CD4^+$ T cells (37); and d) higher levels of TNF-α in active, as well as, resolved lesions (38). In general, ML patients tend to mount exaggerated cell- and Ab-mediated immune responses to self and parasite antigens in the mucosa (39).

From a clinical perspective, management and treatment of ML patients poses a complex public health task, mainly for four reasons. First, ML disease often complicates due of the massive tissue destruction, and its association with secondary bacterial infections, which if left untreated can lead to death (40). Second, common side-effects of ML treatment include severe liver and kidney failure, which often shortens the overall treatment duration period and increase the chances of treatment failure (41). Third, not only drug treatment for ML is significantly more expensive than common treatment for CL, but also few new drugs have been develop in the last 40 years (42). Lastly, parasite drug resistance increases in ML, an event that lowers treatment efficacy rates. Treatment failure during ML treatment has become more notorious during the last few years, when large-scale patient studies have rarely reported successful treatment rates of 70% (42). Collectively, all these reasons make ML a troublesome disease to control, especially in low-income developing countries.

ANIMAL MODELS FOR *L. BRAZILIENSIS* INFECTION

Murine models of leishmaniasis are an important tool that allows the dissection of the pathogenic mechanisms related to disease progression (43). Furthermore, these models also allow the evaluation of factors such as: immune protection, resistance to re-infection, and host genetic risk factors (44). Murine models have been widely used to study the pathogenesis of the Old World species *L. major* in different strains of mice. BALB/c mice are highly susceptible to *L. major* infection so they develop severe and uncontrolled lesions; on the other hand, B6 mice represent a "resistant" disease scenario (45). Infection of B6 mice with *L. major* results in the development of localized, self-healing cutaneous lesions, which generates a protective immunological memory response capable of controlling future challenges (46).

Because the extensive tissue damage observed ML lesions is strongly associated with tissue inflammation, chemokine production may play a role during human ML lesion initiation and expansion. Interestingly, using an animal model of *Lb* infection, it has been shown that different *Lb* clinical isolates (from the same geographic area) can induce different chemokine expression patterns *in vivo* (47). The isolates that induced the largest lesions in B/c mice also induced the highest levels of cell recruitment, without a significant difference in parasite burden. Because chemokines are known for their capacity to induce cell chemotaxis, and during early immune responses are responsible

for recruitment of innate immune cells to sites of inflammation (48), the authors examined the chemokine patterns induced by low and highly pathogenic isolates of *Lb*. The authors concluded that highly pathogenic isolates of *Lb* induce a quick (6 hr p.i.) upregulation at the site of infection of CCL2, CCL3, CCL11 and CXCL10, all highly inflammatory chemokines. This chemokine production was also accompanied by increased transcription rates of the chemokine receptors CCR1, CCR3, CCR5, CXCR3 and CXCR3. Collectively, the above mentioned studies clearly showed that two *L. braziliensis* isolates are capable of inducing different chemokine expression patterns in mice, which may ultimately be implicated in different intensities of disease manifestation in humans. Despite this, no further reports have addressed the impact of *Lb* infection on human cells, or the possible role of chemokines on *Lb*-mediated disease exacerbation. Our experiments on Chapter 4 are intended to answer these questions

Because most inbred strains of mice are naturally resistant to *Lb* infection and present only a transient period of disease, mouse models of *Lb* infection may not truly mimic human disease. For the study of *Lb* infection, hamsters have been reported to be a better model than mice because all laboratory strains of hamsters are susceptible to *Lb* infection, and hamsters sporadically develop ML from initial CL lesions. ML in hamsters occurs through a rare metastatic event that resembles ML incidence in humans. Despite these advantages in using hamsters as an infection model, the paucity of immunological reagents (Abs, cytokines, etc), and the lack gene-specific knockout strains make them difficult to use for in detail immunological studies.

Mouse *Lb* infection is characterized by a small cutaneous lesion that persists for 6 to 8 weeks. The natural "resistance" of most mouse strains is associated with the development of a strong Th1 immune response against the parasite. In the B6 mouse model of *Lb* infection, parasites can be detected for up to 6-8 weeks post infection. Clearance of parasites is associated with elevated levels of IFN-γ and TNF-α in the draining LN (dLN) and increased activation of MΦs which leads to the parasite killing at the site of infection (43). Despite this, the underlying mechanisms and the specific cell involvement during *Lb* infection and posterior parasite clearance remain largely unknown. Contrary to *Lb*, infection with *La* parasites causes progressive disease in all inbred strains of mice (49).

Because of this "disease outcome dichotomy", and the close geographic and genetic resemblance between *La* and *Lb* parasites, we chose to include *La* parasites, as an "infection control" in most of our experiments. Moreover, since our laboratory has extensively studied the regulation of the innate and adaptive immune responses to *La* infection (50-54), its inclusion in my studies offers not only a valuable comparison tool, but also a good technical control. Table 1.2 shows a comparative analysis of the immunological features associated with *La* and *Lb* infection in mice.

Mouse	***L. amazonensis***	***L. braziliensis***
Most tested mouse strains	All susceptible; non-healing lesions	All resistant; self-limited infection
Innate/early responses	Weak & delayed	Strong & T_h1-favored
T_h cell response during infection	Weak, T_h1/T_h2 mixed	Strong T_h1 (IFN-γ, IL-17)
DC responses (*in vitro* evidence)	Impaired DC activation	Strong production of IL-12p40 and IL-10
Parasite Biology		
Parasites in IFN-γ-primed MΦs	Enhanced growth for amastigotes	Efficient parasite killing
Parasites in DC	STAT molecule degradation	Strong STAT1/ISG15 activation

Table 1.3 Differential mouse immune responses to two New World species of *Leishmania.* Animal models for *La* and *Lb* infection resemble different disease outcomes in humans. STAT, signal transducer and activator of transcription; ISG15, IFN-stimulated gene 15.

LEISHMANIA INTERACTION WITH DENDRITIC CELLS AND THE INNATE IMMUNE SYSTEM

DCs are the most efficient APC for priming naïve T cells and are capable of stimulating both innate and adaptive immune responses either by cytokine production or by direct activation of specific T cell subsets (55, 56). In this context, immature DCs, which are located in most non-lymphoid areas of the body, watch for the presence of pathogens and microbial molecules. Upon Ag capture, DCs migrate to lymphoid organs and mature into potent APCs, capable of priming naïve T cells towards a specific cell differentiation pathway (57). In 1993, the first report indicating a direct role for DCs (in this particular case a LC) during the immune response to an invading pathogen was reported by Blank *et al.* (58). These authors reported that *ex vivo* mouse LCs could efficiently ingest *L. major* promastigotes. Furthermore, they concluded that since the parasite number in each infected LC was consistently low, the main task of such cells would be Ag presentation, rather than parasite growth; therefore, strengthening the APC function of these cells (58).

As mentioned above, it is generally thought that tissue DCs initiate polarized immune responses upon antigen capture and migration into the draining LN (59). Recently, Nakano *et al.* have demonstrated that a second pathway for Ag capture also occurs *in vivo*. These researches reported that upon viral infection, monocytes are

recruited from the blood into the lymph nodes and differentiate into potent $CD11c^{+}CD11b^{hi}Gr\text{-}1^{+}$ inflammatory DCs, that produce bioactive IL-12 and promote Th1 immune responses (60). Collectively, these studies suggest that different subpopulations of DCs could be involved in the immune response against different invading pathogens. During *Leishmania* infection, DCs and MΦs are the most important cells for regulating T cell responses and the host's ability to control disease (61). The underlying mechanisms by which the above-mentioned cells contribute to *Lb* infection deserves attention and needs to be determined, therefore our studies on Chapter 2 are aimed at studying the mechanisms of *Lb*-DC interaction.

Interestingly, DCs and MΦs express different sets of pathogen recognition receptors (PRRs), which recognize pathogen-associated molecular patterns (PAMPs) on the surface of invading pathogens. On the basis of their function, PRRs can be classified as endocytic or signaling. Endocytic PRRs are involved in the recognition, uptake and destruction of pathogens and are mainly expressed by phagocytic cells. Examples of endocytic PRRs include the mannose receptor (MR), the glucan receptor, and secreted PRRs such as complement receptors, mannan-binding lectins, C-type lectin receptors (such as DC-SIGN and L-SIGN), and peptidoglycan binding proteins (62, 63). On the other hand, signaling PRRs recognize PAMPs and induce a specific signaling cascade that can lead to the production of cytokines and the activation of the host's defense mechanisms. Examples of signaling PRRs include Toll-like receptors (TLRs), cytosolic Nod-like receptors (NLRs) and RNA helicases (62, 64).

TLRs, which were initially described by Medzhitov *et al.* in 1997, play a crucial role in the early recognition of bacterial, fungal, viral and parasitic PAMPs (64, 65). Over the years, many different PAMPs have been identified and associated with their respective TLRs. A few examples of these PAMP/TLR interactions include: peptidoglycan (TLR2), double-stranded RNA (TLR3), lipopolysaccharide (TLR4), flagellin (TLR5), single-stranded RNA (TLR7), G-rich oligonucleotides (TLR8), and methylated bacterial CpG-DNA motifs (TLR9) (64, 66). TLRs can also act cooperatively in order to recognize different modifications in PAMPs. For example, TLR2/1 heterodimers recognize triacilated lipoproteins, whereas TLR2/6 heterodimers recognize diacylated lipoproteins, respectively (67). Based on their cellular localization, TLRs can be further classified as intracellular TLRs (such as TLRs 3, 7, 8 and 9), or extracellular TLRs (such as TLRs 2/1. 2/6, 4 and 5).

Upon interaction between a TLR and its respective PAMP, three signaling cascades can possibly occur. Most TLRs use the adaptor protein MyD88 (also called the canonical signaling pathway for TLRs), which interacts with different serine/threonine kinases (in most cases IRAK1, IL-1-receptor-associated kinase) to propagate the signal. MyD88-associated downstream signaling continues through the TNF-receptor associated factors (TRAF) family and finally leads to the activation of the NF-κB and MAPK transcription factors which activate the transcription of the relevant immune-related genes (62). The second adaptor molecule used during TLR signaling is called TIR-associated protein (TIRAP, also known as Mal). Mal-deficient mice were shown to have impaired

NF-κB and MAPK activation after TLR1, TLR2, TLR4 and TLR6 ligation, suggesting a direct role for this adapter protein in TLR signaling (68). However, DC maturation after LPS exposure remained intact in $Mal^{-/-}$ mice, which in turn suggested the existence of a third MyD88- and Mal-independent pathway (69). A TLR3-based, yeast two-hybrid system led to the discovery of the third adaptor protein known as TRIF (TIR-domain-containing adapter-inducing IFN-β). Upon TLR3 ligation, TRIF induces the production of IFN-β by activation of the IFN-regulatory factor 3 (IRF3) (70, 71).

Recently, particular attention has been directed towards the role of different TLRs during *Leishmania* infection. *L. major* lipophosphoglycan (LPG) has been found to directly activate NK cells through TLR2 (72). Furthermore, by using RNA interference, Flandin and colleagues found that both TLR2 and TLR3 are involved in the recognition and killing of *L. donovani* promastigotes by IFN-γ-primed MΦs (73). DCs are known to express the widest TLR repertoire among immune cells, and different TLR ligands have been shown to potentiate the connection between pathogen Ag recognition and the downstream induction of T cell immunity (Fig. 1.4) (74). Given that *Lb* infection involves a strong initial innate immune response, we speculate that parasite recognition by DCs through TLR2 and MyD88-associated pathways may influence the downstream responses important for parasite clearance. Our studies in Chapter 3 are specifically designed to test this hypothesis.

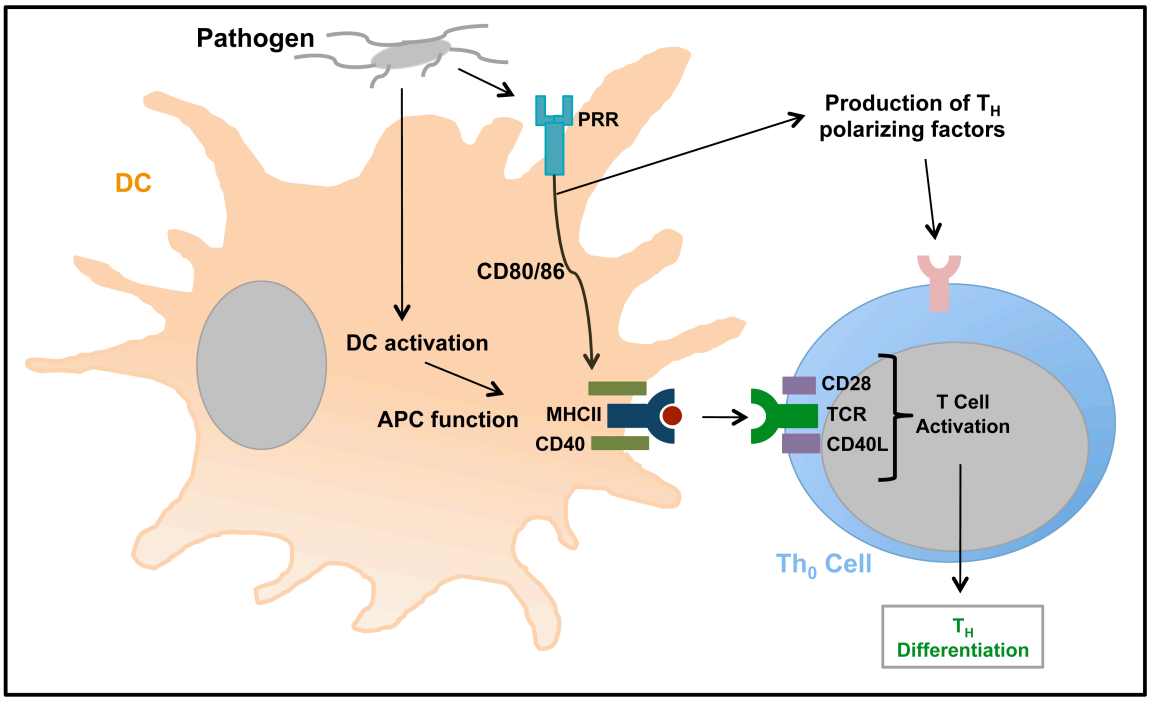

Figure 1.4 DC-T cell interactions and the induction of a polarized immune response. Effective T cell activation by DCs requires 3 main signals: 1) Antigen presentation through the MHCII/TCR complex, 2) Triggering of CD40L and CD28 by DC co-stimulatory signals such as CD40 and CD80/86, and 3) Activation induced by soluble or membrane-bound polarizing factors such as IL-12 and CCL2. *Partially adapted from* (5).

LEISHMANIA'S STRATEGIES FOR IMMUNE EVASION

The evolutionary persistance (~300 million years) of trypanosomatid parasites such as *Leishmania* and *Trypanosoma* suggests that these parasites must have evolved numerous strategies in order to survive within their host organisms (75). Possibly the most important strategy used by *Leishmania* parasites to avoid immune recognition by the host is their capacity to "silently infect" cells. It has been previously reported that *Leishmania* pro- and amastigotes can enter DCs and MΦs without activating or inducing maturation events in these cells (76). Furthermore, Yang *et al.* described that even at high infection ratios (up to 20:1), MΦs infected with *La* did not induce the production of IL-12 and IL-10 (77). Despite this lack of cytokine induction by *La*, infection did induce the activation of the mitogen-activated protein kinase (MAPK) and the extracellular signal-regulated kinases (ERK) pathways. However, this was not sufficient to elicit cytokine production. *Leishmania* parasites can also evade complement-mediated lysis, and at the same time use C3b complement coating as a mechanism to target MΦs. When exposed to C3b, surface molecules in the metacyclic promastigote coat prevent the further binding of the C5b-C9 complexes, therefore preventing formation of the membrane attack complex (78). However, the already attached C3b proteins can be cleaved by the *Leishmania* surface proteinase gp63 into the inactive form iC3b, which favors silent entry into MΦs (79).

Even though "silent entry" is an important mechanism used by *Leishmania* parasites, it occurs only during the early stages of infection, when parasite-specific Abs are not present. Woelbing *et al.* reported that IgG-coated parasites (either pro- or amastigotes) can induce higher levels of DC maturation than non-coated parasites, suggesting that Ab-coating of *Leishmania* parasites can potentially induce stronger immune responses (56). Despite this, studies performed in our laboratory have described a pathogenic role for B cells during *La* infection, mainly because parasite-specific Ab can enhance parasite recognition by APCs and therefore promotes the priming of pathogenic T cells (80).

The second immune evasive strategy used by *Leishmania* parasites involves the suppression of the host immune responses. *Leishmania* parasites do not release toxins while interacting with host cells (81). Therefore, unlike other intracellular bacterial pathogens, they survive by modulating the host's immune functions. Despite the fact that immune responses against *Leishmania* parasites are species-specific, all *Leishmania* species interfere with the host's defense mechanisms to various degrees. For example, the highly immunosuppressive species *La* can efficiently prevent the up-regulation of pro-inflammatory cytokines in infected DCs and MΦs (82). Particularly in MΦs, *L. major* parasites are known to interfere with the nitric oxide (NO) pathway through the upregulation of anti-inflammatory cytokines such as IL-10 and TGF-β (83, 84). Similarly, upon *La* and *L. mexicana* infection, MHC class II molecules are recruited to the PV, where they can be degraded by parasite-derived cysteine proteases (85, 86).

Furthermore in DCs, *La* interferes with the JAK/STAT signaling pathway, preventing IL-12 production and Th1 differentiation, thus hindering the induction of protective immune responses and favoring disease progression (50, 87). Likewise, less immunosuppressive species such as *Lb* are also capable of causing degradation in these pathways, although to a lesser degree (88). Whether or not these different levels of suppression contribute to disease severity *in vivo* is a matter of continuous research.

OBJECTIVES OF THIS DISSERTATION

The limited information available describing the mechanisms by which mice effectively clear *Lb* infection do not permit a correlation to other non-healing *Leishmania sp.* models. Thus, detailed study of the protective immune response against *Lb* in mice will help identify new therapeutic targets that could be exploited to overcome infection by other *Leishmania sp.* Knowing that DCs play a crucial role in regulating T cell differentiation, *Lb*-DC interactions will be examined in Chapter 2 in order to determine the contribution of these interactions on the establishment of a protective immune response. During *Lb* infection, the specific set of $CD4^+$ T cells activated by *Lb*-infected DCs has not been studied; therefore its identification will help determine the factors involved in disease control. DCs express different PRRs on their surface, and activation of these receptors by microbial components can greatly influence the downstream T cell function. The interaction between *Lb* parasites and components of the TLR signaling

pathway has not yet been studied; therefore, the contribution of MyD88 and TLR2 to DC activation, and to the downstream priming of a protective T cell response will be evaluated in Chapter 3.

Given that *Lb* is the causative agent of ML in humans, one of the main objectives of this dissertation is to use an *in vitro* system to study the human immune response against *Lb*. In Chapter 4, we analyze in detail the interactions between PBMCs and *Lb* parasites, focusing on the induction of proinflammatory mediators and their potential contribution to disease exacerbation during ML. Collectively, this dissertation is intended to further improve the understanding of *Lb* interactions with the host immune system.

CHAPTER 2: *LEISHMANIA BRAZILIENSIS* INFECTION INDUCES DENDRITIC CELL ACTIVATION, ISG15 TRANSCRIPTION AND THE GENERATION OF PROTECTIVE IMMUNE RESPONSES[1]

INTRODUCTION

Leishmaniasis is a vector-transmitted disease, and at least 20 species of *Leishmania* can cause human infections (6). *Leishmania* are dimorphic intracellular parasites that survive and multiply as flagellated promastigotes in the midgut of sand flies, and as non-flagellated amastigotes in mammalian MΦs and DCs (14, 89). *Lb* is a causative agent of CL in Latin America and the main cause of ML (90). ML is a severe and disfiguring form of the disease, usually compromising mucosal regions of the nose, mouth and pharynx (38, 39). In humans, ML is characterized by excessive T and B cell responses to the parasite, with elevated levels of anti-*Leishmania* antibodies and excessive production of IFN-γ and TNF-α (32, 91). On the other hand, *Lb* infection is self-healing in most inbred strains of mice, presenting only a transient period of active disease in the skin (92, 93). It is known that the clearance of *Lb* in mice is primarily due to an elevated production of IFN-γ by T cells and MΦs, both at the site of infection and in the draining lymph nodes (LNs) (43, 94).

[1] Published in *Journal of Immunology*, 180(11):7537-45, 2008, with minor modifications. Copyright 2008. The American Association of Immunologists, Inc.

Modulation of DC activation by *Leishmania* parasites appears to be species-specific (95). For example, some species (*L. major* and *L. donovani*) are capable of activating DCs to express co-stimulatory molecules on the surface and to produce IL-12, in the presence of IFN-γ and anti-CD40 mAb (56, 96). By contrast, New World species such as *La* and *L. mexicana* either fail to activate DCs or cause an impaired DC response to secondary stimuli, such as LPS and IFN-γ (51, 97). DCs are potent APCs that can stimulate both the innate and adaptive immune systems, either by producing cytokines or by directly activating specific subsets of effector T cells (49, 51). The role of DCs in the immune response against *Leishmania sp.* infection has been characterized in several healing and non-healing models, and the importance of these specialized cells in adaptive immune responses is evident (49, 51, 56). Intracellular signaling events involved in *Leishmania* infection have been described in infected MΦs, where several *Leishmania* species can repress the JAK/STAT and MAP kinase signaling pathways (98) and alter antigen-presenting capabilities and cytokine production in the infected cells (99). At present, there is no report describing the interactions between *Lb* parasites and DCs (15).

In the present study, we generated axenic amastigotes of *Lb* and evaluated the activation/maturation events triggered by infection in DCs, and its consequences on T cell responses. The use of *La*, another member of the New World species, as a control allowed us to define molecules and T cell populations that were selectively up-regulated during *Lb* infection *in vitro* and *in vivo*. Using fluorescence-labeled pro- and amastigotes,

we demonstrated that *Lb* infection induced DC activation/maturation and IL-12p40 production in both infected and bystander cells, and that DC activation was accompanied by up-regulation of the JAK/STAT signaling pathway, especially in regard to the expression of p-STAT1, p-STAT3, IRF-1, and ISG15. Moreover, *Lb*-infected DCs were highly efficient in priming naïve $CD4^+$ T cells to proliferate and to produce IFN-γ and IL-17. We, therefore, propose that a rapid induction and fine regulation of IFN-γ-producing $CD4^+$ T cells can be attributed to the control of *Lb* infection.

MATERIALS AND METHODS

Mice: Female B6 and BALB/c mice (Harlan Sprague Dawley, Indianapolis, IN) were used in this study. Mice were maintained under specific pathogen-free conditions and used for experimentation at 6 to 8 weeks of age, according to protocols approved by the UTMB Institutional Animal Care and Use Committees.

Parasite culture and Ag preparation: Infectivity of *Lb* (strains LTB111 and LC1418) was maintained by regular passage through Syrian golden hamsters (Harlan Sprague Dawley). Infectivity of *La* (strain LV78, clone 12-1; a gift from Kwang-Poo Chang, Departments of Microbiology and Immunology, Chicago Medical School) was maintained by regular passage through BALB/c mice. All promastigotes were cultured at 23°C in Schneider's *Drosophila* medium (Invitrogen, Carlsbad, CA), pH 7.0,

supplemented with 20% FBS (Sigma, St. Louis, MO), 2 mM L-glutamine, and 50 μg/ml gentamicin. Stationary promastigote cultures of less than five passages were used for DC or animal infection. Axenic amastigotes of *Lb* (LC1418) and *La* (LV78) were cultured at 33°C in complete Grace's insect cell culture medium (Invitrogen), pH 5.0, supplemented with 20% FBS. *Lb* axenic amastigotes required 5% CO_2 for their growth. To prepare promastigote lysates, parasites (2×10^8/ml) were subjected to six freeze-and-thaw cycles in liquid nitrogen and a 15-min sonication. Ag preparations were stored in aliquots until use.

DC generation and infection: BMDCs were generated from B6 mice in complete IMDM (Invitrogen) containing 10% FBS, supplemented with 20 ng/ml recombinant GM-CSF (eBioscience, San Diego, CA), and harvested at day 8. Parasites (5×10^7/ml in PBS) were labeled with CFSE (5 μM, Sigma) at room temperature for 5 min. The efficiency of parasite labeling was >99%, as judged by FACS and microscopic examination. Labeled parasites were washed four times and incubated with DCs (8:1 parasite-to-cell ratio) at 33°C for 12 h, and then at 37°C for another 12 h. LPS (100 ng/ml) from *Salmonella enterica* serovar Typhimurium (Sigma) plus IFN-γ (100 ng/ml, Leinco Technologies, St. Louis, MO) was used as a positive control in all of the experiments. At 24 h post-infection (p.i.), cells were collected for FACS analysis and extracted for RNA or proteins, and culture supernatants were harvested for cytokine detection.

DC sorting and immunofluorescence: A total of 4×10^6 DCs infected with CFSE-labeled *Lb* parasites were sorted, based on their FL1 fluorescence with a FACSaria Cell Sorter (BD Biosciences). $CFSE^+$ and $CFSE^-$ DCs were then cytospun onto glass slides, counterstained with mounting medium containing 4′,6′-2-diamidino-2-phenylindole (DAPI, Vector Laboratories, Burlingame, CA), and assessed for infection status under an Olympus BX51/52 fluorescence microscope (Olympus America Inc., Melville, NY). The percentage of infected cells in each sorted subpopulation was determined by counting infected and uninfected cells using a total of 10 pictures obtained from random visual fields. The percentage of infection was calculated as the number of infected cells / total number of cells x 100.

T cell priming *in vitro*: Naïve $CD4^+$ T cells were purified from the spleen of B6 mice by negative selection using magnetic beads (Miltenyi Biotec, Alburn, CA), and their purity was routinely around 95%, as judged by CD4 staining and FACS analysis. Purified $CD4^+$ T cells (2×10^5) were co-cultured with parasite-infected, mitomycin C-pretreated DCs at a 10:1 ratio in 96-well plates for 3 days. Supernatants were harvested for cytokine detection. To assess T cell proliferation, 1 μCi of [^{3}H]thymidine was added at 18 h before harvest, and incorporated radioactivity was determined on a microplate scintillation and luminescence counter (Packard Instrument Company, Downers Grove, IL). Naïve $CD4^+$ T cells were also co-cultured with sorted $CFSE^+$ and $CFSE^-$ DCs using the same protocols.

Intracellular staining and FACS analysis: The following specific mAbs and their corresponding isotype controls were purchased from eBiosciences: FITC-conjugated anti-IFN-γ (XMG1.2), anti-CD45RB (C363.16A), and rat IgG2a; PE-conjugated anti-IL-17A (eBio17B7), anti-IL-10 (JES5-16E3), anti-IL-12/IL-23p40 (C17.8), anti-CD40 (1C10), anti-CD80 (16-10A1), anti-CD83 (Michel-17), as well as rat IgG1, IgG2a, and IgG2b; PE-Cy5-conjugated anti-CD4 (GK1.5), anti-CD11c (N418), and hamster IgG. Briefly, cells were washed, blocked with 1 μg/ml FcRγ blocker (CD16/32, eBioscience), stained for specific surface molecules, fixed/permeabilized with a Cytofix/Cytoperm Kit (BD Biosciences, Franklin Lakes, NJ), and then stained for specific intracellular molecules. To detect intracellular cytokines, 1 μl/ml of Golgi Stop (BD Biosciences) was added for the last 6 h of cultivation. Cells were read on a FACScan (BD Biosciences) and analyzed using FlowJo V8.5 Software (TreeStar, Ashland, OR).

Cytokine ELISA: The levels of cytokines in culture supernatants were measured using ELISA kits purchased from BD Biosciences (IL-12p40 and IL-10) or eBioscience (IFN-γ and IL-17). Detection limits were 15 pg/ml for IFN-γ, 4 pg/ml for IL-10, 10 pg/ml for IL-12p40, and 8 pg/ml for IL-17, respectively.

JAK/STAT microarray and IL-12p40 real-time RT-PCR: Total RNA was extracted from 1 x 10^6 to 2 x 10^6 DCs using the RNeasy system (QIAGEN, Valencia, CA). Genomic DNA was digested with the on-column RNase-free DNase (QIAGEN). Total RNA (10 μg) was subjected to Oligo GEArray analyses (SuperArray Bioscience

Corporation, Frederick, MD) for the JAK/STAT signaling pathway. For detecting IL-12 transcripts, cDNA was synthesized from 1 μg of total RNA using Superscript II reverse transcriptase (Invitrogen) primed with oligo(dT). Real-time RT-PCR was performed at UTMB's institutional Real-Time PCR core facility, and all reagents were purchased from Applied Biosystems (Foster City, CA): 20× assay mixture of primers, TaqMan MGB probes (FAM dye-labeled) for mouse IL-12p35 and IL-12p40 (P/N 4331182), pre-developed 18S rRNA (VIC dye-labeled, as an endogenous control), and TaqMan assay reagent (P/N 4319413E). Separate-tube (singleplex), real-time PCR was performed with 40 ng cDNA for both target genes and the endogenous control, using a Taqman Gene expression master mix (P/N 4370074). The PCR cycling parameters were: UNG activation at 2 min 50°C; AmpliTaq activation 95°C for 10 min; denaturation 95°C for 15 sec; and annealing/extension 60°C for 1 min (repeat 40 times) on ABI7000 (Applied Biosystems). Duplicate CT values were analyzed in Microsoft Excel using the comparative CT(ΔΔCT) method, as described by the manufacturer (Applied Biosystems). The amount of target ($2^{-\Delta\Delta CT}$) was obtained by normalized-to-endogenous reference (18s) and relative to uninfected control samples.

Western blot: A total of 4×10^6 DCs were infected with parasites at a 10:1 parasite-to-cell ratio. At 6 h p.i., some groups were stimulated with either LPS (100 ng/ml) plus IFN-γ (100 ng/ml) or LPS (100 ng/ml) plus IFN-α (100 U/ml, Cell Sciences, Canton, MA) for 30 min before protein extraction. Cells were lysed in ice-cold RIPA lysis buffer containing complete EDTA-free protease and phosphatase inhibitors (Pierce

Biotechnology, Rockford, IL). Protein concentrations were determined using the BCA protein assay kit (Pierce Biotechnology). Equal amounts of proteins were loaded onto SDS-polyacrylamide gels, which were either a 10% gel (to detect STATs and IRFs) or 15% (to detect ISG15), and then transferred to polyvinylidene difluoride membranes (BioRad Laboratories, Hercules, CA). Rabbit anti-mouse STAT1, p-STAT1, STAT2, STAT3, p-STAT3, IRF-1, ISG15 molecules, as well as HRP-conjugated goat anti-rabbit IgG and HRP-conjugated goat anti-mouse IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-mouse IRF-3 and p-STAT2 were purchased from Cell Signaling Technology (Danvers, MA) and Upstate Biotechnology (Charlottesville, VA), respectively. Mouse anti-actin mAb (Sigma) was obtained from Dr. Jiaren Sun (Department of Microbiology and Immunology, UTMB, TX). Membranes were incubated with primary Abs [diluted 1:2,000 except for anti-ISG15 (1:500) in TBS buffer containing 5% nonfat milk and 0.05% Tween-20] at 4°C overnight, washed, and incubated with HRP-conjugated secondary Abs (1:3,000) for 1 h. Blots were developed with the enzyme chemiluminescence kit ECL (Amersham Biosciences, Piscataway, NJ). Densitometry analysis of blots was performed using AlphaEase FC Stand-Alone Software (version 4.0, Alpha Innotech Co., San Leandro, CA).

Evaluation of infection and T cell activation: Metacyclic promastigotes of *Lb* and *La* were purified according to a previously described protocol (100) and injected s.c. in the right hind foot of B6 mice (2×10^6 parasites/mouse, 5 mice/group). Lesion sizes were monitored weekly with a digital caliper (Control Company, Friendswood, TX), and tissue

parasite burdens were measured via a limiting dilution assay using LDA software (Oxford University Press, New York, NY) (101). At 4 and 8 wk p.i., mice were euthanized, and the popliteal LN cells were collected and stained immediately for the expression of activation markers on $CD4^+$ T cells. Some LN cells (3×10^6/well/ml) were stimulated with PMA/Ionomycin/Golgi Plug in the absence of parasite Ag for 6 h (*ex vivo*) prior to examination of intracellular IFN-γ, IL-17 and IL-10 expression.

Statistical analysis: The difference between two different groups was determined by the Student's *t*-test. One- or two-way ANOVA was used for multiple group comparisons (GraphPad Software v4.0, San Diego, CA). Statistically significant values are referred to as follows: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

RESULTS

L. braziliensis axenic amastigotes have enhanced infectivity and induce high levels of IL-12p40 secretion in DCs

Although there is increasing evidence for a marked suppression of DC function by *La* and *L. mexicana* amastigotes (49, 51, 97), we find no reports in the literature as to how DCs respond to *Lb* amastigotes. To address this issue, we generated short-term axenic amastigotes for *Lb* and first examined their infectivity in DCs. After exposing DCs

to two different doses (4:1 and 8:1 parasite-to-cell ratios) of CFSE-labeled *Lb* pro- and amastigotes for 24 h, we consistently observed significantly higher percentages of $CD11c^+$ DCs that were infected with amastigotes when compared to those infected with promastigotes (Fig. 2.1A). To assess the function of these infected DCs, we included cells infected with *La* as a control and tested several infection doses (2:1, 4:1, and 8:1). As shown in Fig. 2.1B, while both promastigotes were capable of inducing IL-12p40 secretion in DCs, *Lb* promastigotes were more potent in doing so than their *La* counterparts ($p<0.05$ in all three tested doses). In sharp contrast to the general failure of *La* amastigotes in activating DCs (49, 51), *Lb* amastigotes were highly competent in activating DCs and tended to stimulate higher (6 ng/ml) levels of IL-12p40 than did their promastigote counterparts (4 ng/ml) at an 8:1 infection dose ($p<0.05$). These results suggested remarkable differential responses of murine DCs to two New World *Leishmania* species, and prompted us to examine the molecular basis underlying these differences.

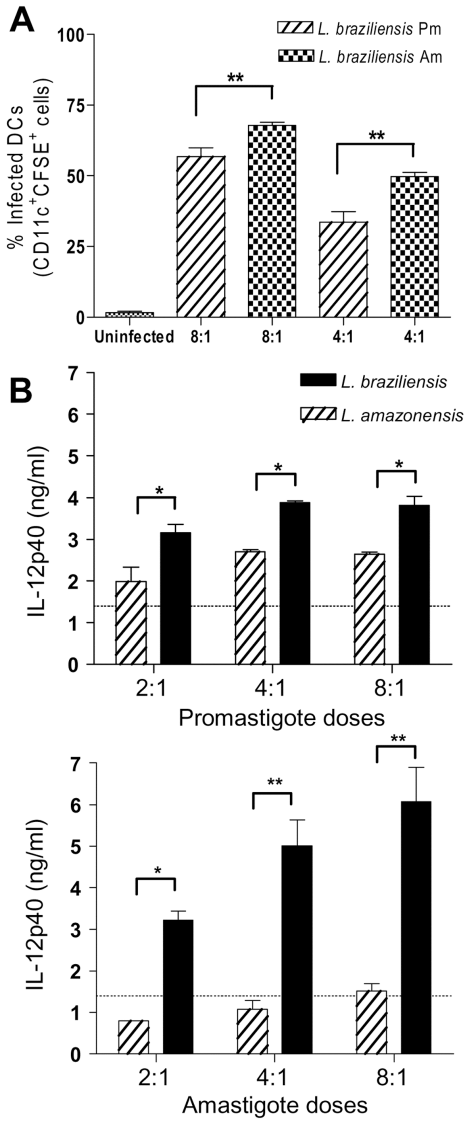

Figure 2.1 *L. braziliensis* axenic amastigotes are highly efficient in activating DCs. BMDCs derived from B6 mice were infected with pro- (Pm) and amastigotes (Am) of *Lb* or *La* at the indicated parasite-to-cell ratios for 24 h. (A) CFSE-labeled parasites were used to infect DCs and measure the percentage of infected cells (gated on CD11c and CFSE). (B) IL-12p40 production in the culture supernatants of Pm or Am-infected DC was assayed by ELISA. Dotted line represents baseline production by un-infected DCs (1.40 ± 0.1 ng/ml). Shown are representative results from three independent repeats. Bars represent statistically significant differences between the two *Leishmania* species. *($p<0.05$) and **($p<0.01$) indicate statistically significant differences between the control and infected groups by one-way ANOVA analysis.

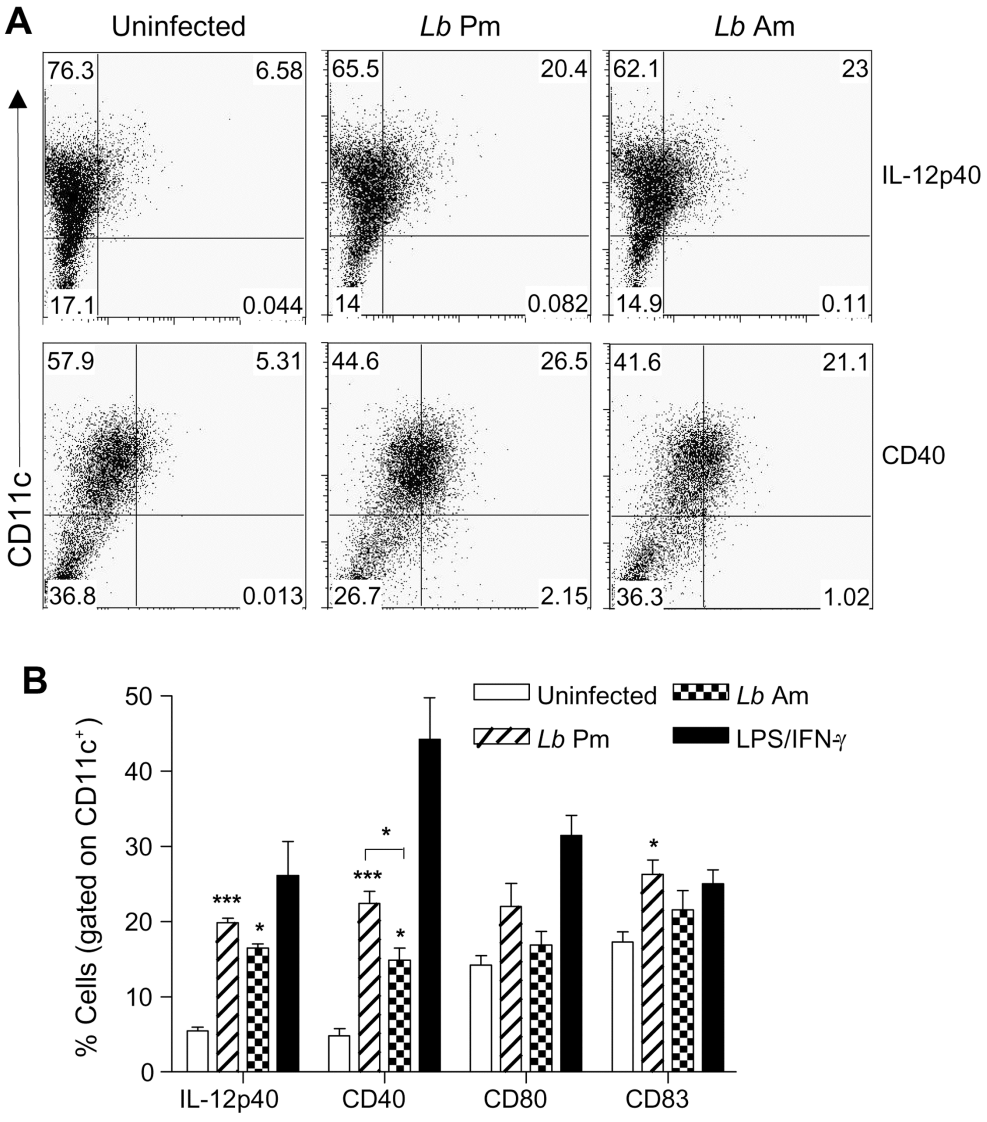

Figure 2.2 *L. braziliensis* promastigotes and amastigotes induce DC maturation and activation. BMDCs were infected with *L. braziliensis* parasites (Pm and Am, 8:1 parasite-to-cell ratio) for 24 h. DCs treated with LPS (100 ng/ml) plus IFN-γ (100 ng/ml) were included in all experiments and served as positive controls. (A) Expression of intracellular IL-12p40 and surface maturation markers (CD40, CD80 and CD83) was assayed by FACS. Shown are representative results from four independent repeats. (B) Data from four repeats are pooled and shown in the plot. Bars represent statistically significant differences between Pm and Am. *($p<0.05$) and ***($p<0.001$) indicate statistically significant differences between the control and infected groups by one-way ANOVA analysis.

DC maturation is induced after *L. braziliensis* infection

To examine the status of DC maturation/activation, we measured the expression of surface markers (CD40, CD80, and CD83) and intracellular molecules (IL-12p40) at the single-cell level at 24 h p.i. with *Lb* parasites. The distribution of IL-12p40 and CD40 among $CD11c^+$ DCs is shown as an example of FACS plots in Fig. 2.2A, and the results from the pooled data from four independent repeats are given in Fig. 2.2B. DCs stimulated with LPS/IFN-γ were included in all tests as positive controls for general quality/responsiveness of DCs. As compared with non-stimulated DCs, it was evident that infection with *Lb* promastigotes stimulated DCs to express significantly higher levels of IL-12p40, CD40, and CD83 ($p<0.01$ and $p<0.05$), whereas significant increases were detected for IL-12p40 and CD40 following infection with *Lb* amastigotes ($p<0.05$). Consistent with the data shown in Fig. 2.1, both developmental forms of *Lb* parasites induced higher levels of DC activation than did their *La* counterparts (data not shown). Together, these FACS studies confirmed and extended the ELISA data, indicating the ability of *Lb* to activate DCs.

DC activation during *L. braziliensis* infection occurs in both parasite-carrying and bystander cells

Given that approximately 55~70% $CD11c^+$ DCs carried $CFSE^+$ parasites (Fig. 2.1A), we wondered whether the observed DC responses were due to infection or to bystander

activation. To address this issue, we infected DCs with CFSE-labeled *Lb* parasites and analyzed the activation status of DCs between the parasite-carrying ($CD11c^+CFSE^+$) and bystander ($CD11c^+CFSE^-$) cells. As shown in Fig. 2.3A, comparable percentages (3.6% vs. 4.6%) of IL-$12p40^+$ cells were detected in $CD11c^+CFSE^+$ and $CD11c^+CFSE^-$ subsets of DCs at 24 h of infection with promastigotes; however, it appeared that higher percentages (8.1%) of IL-$12p40^+$ cells were detected in $CD11c^+CFSE^+$ DCs following infection with amastigotes than in $CD11c^+CFSE^-$ DCs (2.5%). Similar trends were observed when DCs were infected with parasites for 6 h and then treated with LPS/IFN-γ for an additional 18 h (Fig. 2.3A).

To ensure that the CFSE labeling accurately reflected parasite infection, we sorted the $CFSE^+$ and $CFSE^-$ subsets by flow cytometry (Fig. 2.3B) and assessed the percentages of infected cells under a fluorescence microscope (Fig. 2.3C). Having confirmed the CFSE-labeling protocol, we then quantified the percentages of $CFSE^+$ and $CFSE^-$ subsets among IL-$12p40^+CD11c^+$ and $CD40^+CD11c^+$ DCs when cells were infected with labeled parasites in the presence or absence of LPS/IFN-γ. Data from three independent repeats indicated that comparable levels of DC activation were achieved in parasite-carrying and bystander DCs, and that both DC subsets responded to secondary stimuli comparably (comparing $CFSE^-$ and $CFSE^+$ groups in Fig. 2.3D).

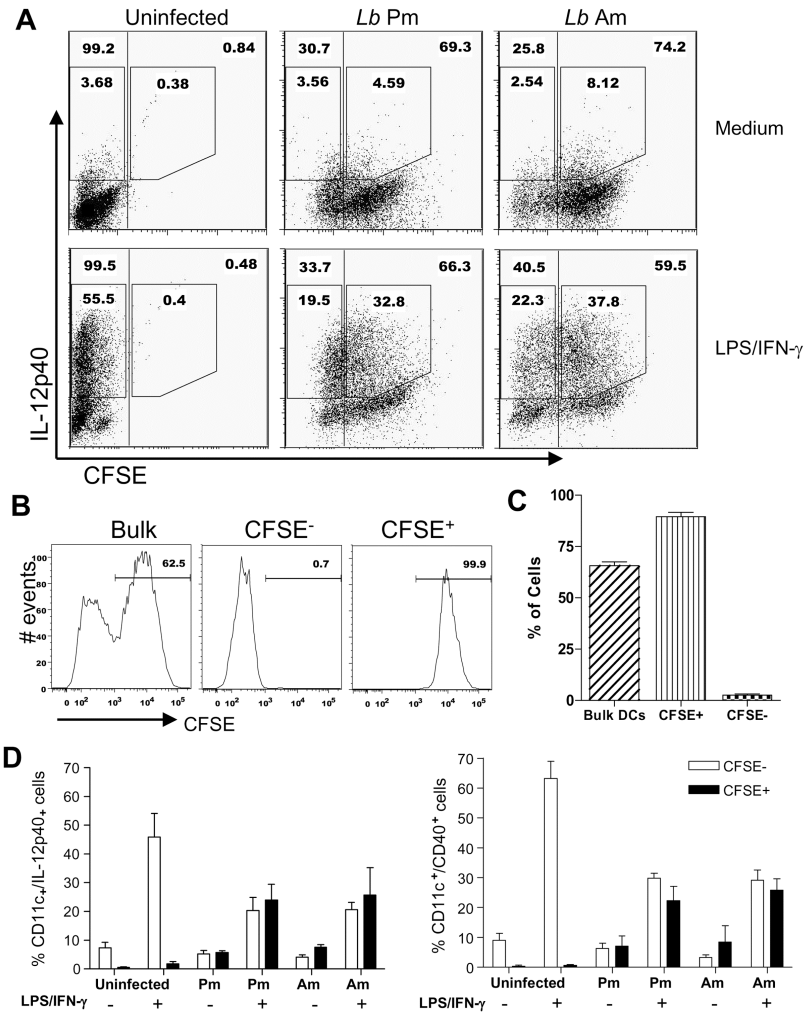

Figure 2.3 Both parasite-carrying and bystander DCs become activated upon *L. braziliensis* infection, produce IL-12p40, and are capable of responding to secondary stimulation with LPS and IFN-γ. BMDCs were infected with CFSE-labeled *Lb* parasites (Pm and Am, 8:1 parasite-to-cell ratio) for 24 h in the absence or presence of LPS/IFN-γ. (A) Intracellular IL-12p40 in both *Lb*-infected ($CFSE^+$) and bystander ($CFSE^-$) DCs was measured by FACS. (B and C) Infected DCs were enriched via a FACSAria Cell sorter and analyzed for the correlation between CFSE fluorescence and detection of the parasite. (B) The purity of $CFSE^+$ and $CFSE^-$ DCs was determined by FACS analysis. (C) The percentages of infected cells in each sorted subpopulation were determined by fluorescent microscopy counting. (D) Intracellular IL-12p40 production and expression of CD40 on the surface of $CFSE^-$ and $CFSE^+$ DCs were determined by FACS analysis and graphed to show comparable responses in both types of DCs. Shown are representative results from three independent repeats.

Transient IL-12p40 gene expression and the activation of the JAK/STAT signaling pathway in *L. braziliensis*-infected DCs

DC responsiveness to different pathogens varies greatly in intensity and duration (102, 103). Since biologically functional IL-12p70 is composed of p40 and p35 heterodimers, we evaluated the kinetics of IL-12 gene expression during *Lb* infection via real-time RT-PCR. As shown in Fig. 2.4A, there were 43- and 56-fold increases in the levels of IL-12p40 mRNA at 8 h of infection with *Lb* pro- and amastigotes, respectively. The expression levels, however, were below 4-fold in both groups at 24 h of infection, suggesting a transient expression of the IL-12p40 gene during *Lb* infection. The levels of IL-12p35 mRNA were below the detection limit at both time points (data not shown).

To understand the intracellular events triggered at the early stage of infection, we examined the JAK/STAT signaling pathway by an Oligo GEArray analysis. Side-by-side comparison of the gene expression profiles revealed that nearly 55 out of 120 tested genes were up-regulated in DCs at 24 h p.i with *Lb* and *La* promastigotes, respectively (data not shown). In comparison to the *La*-infected and uninfected controls, there was an increased transcription of STAT1, STAT2 and nitric oxide synthase 2 (Nos2) in *Lb*-infected DCs (Fig. 2.4B). Notably, *Lb* infection triggered a 23-fold increase in the transcription of ISG15 (Fig. 2.4B), a molecule known to be important in viral infection (104, 105). Semi-quantitative RT-PCR analyses confirmed the up-regulation of STAT1, STAT2, STAT3, and ISG15 genes (data not shown).

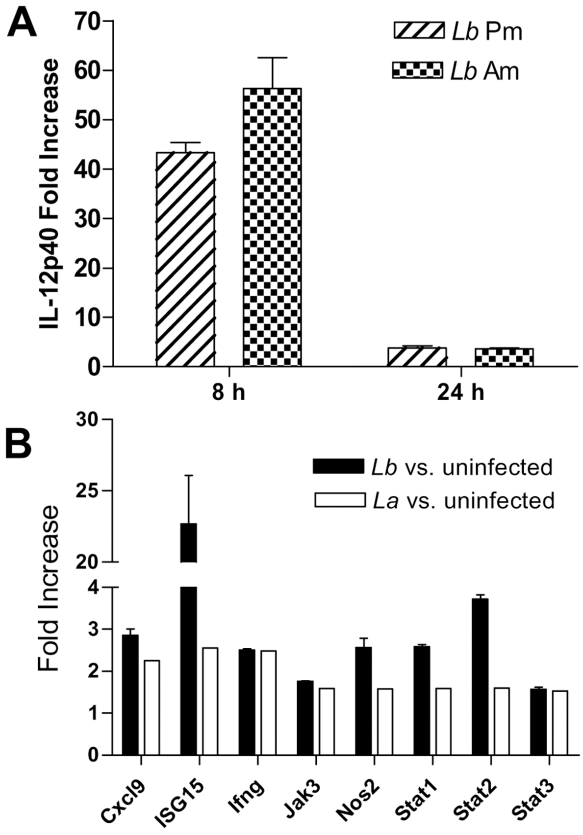

Figure 2.4 DC activation following *L. braziliensis* infection is transient and accompanied by up-regulation of STAT molecules. BMDCs were infected with *Lb* (Pm and Am, 8:1 parasite-to-cell ratio) for 8 or 24 h before RNA extraction. (A) IL-12p40 expression was quantified by real-time RT-PCR. (B) RNA extracted at 24 h was used for Microarray analysis of the JAK/STAT signaling pathway. RNA extracted from *La*-infected BMDCs under similar conditions was used for establishing controls. Shown are representative results from two independent experiments.

L. braziliensis infection up-regulates phosphorylated STAT molecules and the expression of ISG15 proteins

To investigate whether the increased gene expression correlated to total or phosphorylated protein levels, we infected DCs with *Lb* for 6 h and then stimulated cells with LPS/IFN-γ for 30 min prior to protein extraction and Western blot analyses. We found that infection with *Lb* promastigotes resulted in a 2-fold increase in the levels of p-STAT1, and that infection with pro- and amastigotes led to 1.5- to 2-fold increases, respectively, in p-STAT3 (Fig. 2.5A-B). *Lb* infection did not cause major changes in the levels of STAT1, STAT2, p-STAT2, STAT3 (Fig. 2.5A), STAT4 and p-STAT4 (data not shown). Consistent with the microarray data, infection with *Lb*, but not *La*, pro- and amastigotes induced a 3.5-fold increase in the levels of free ISG15 (comparing lanes 5 and 6 with lanes 3 and 4, respectively, Fig. 2.5C). Since ISG15 expression can be modulated through type I IFN signaling (106), we also evaluated the expression levels for IFN regulatory factors 1 and 3 (IRF-1 and IRF-3). IRF-1 and IRF-3 are members of the IRF family of transcription factors that upon nuclear translocation activate the transcription of alpha and beta interferons. Infection with *Lb* pro- and amastigotes resulted in 1.5- and 1.9-fold increases, respectively, in the expression of IRF-1 (Fig. 2.5D). Of note, down-regulation of IRF-1 below the baseline level was observed in *La* amastigote-infected DCs (Fig. 2.5C, lane 4). No major changes were detected for IRF-3 (Fig. 2.5C). Together, these results suggested that *Lb* infection selectively up-regulates

DC signaling pathways known to be critical for innate immune responses and the production of proinflammatory cytokines.

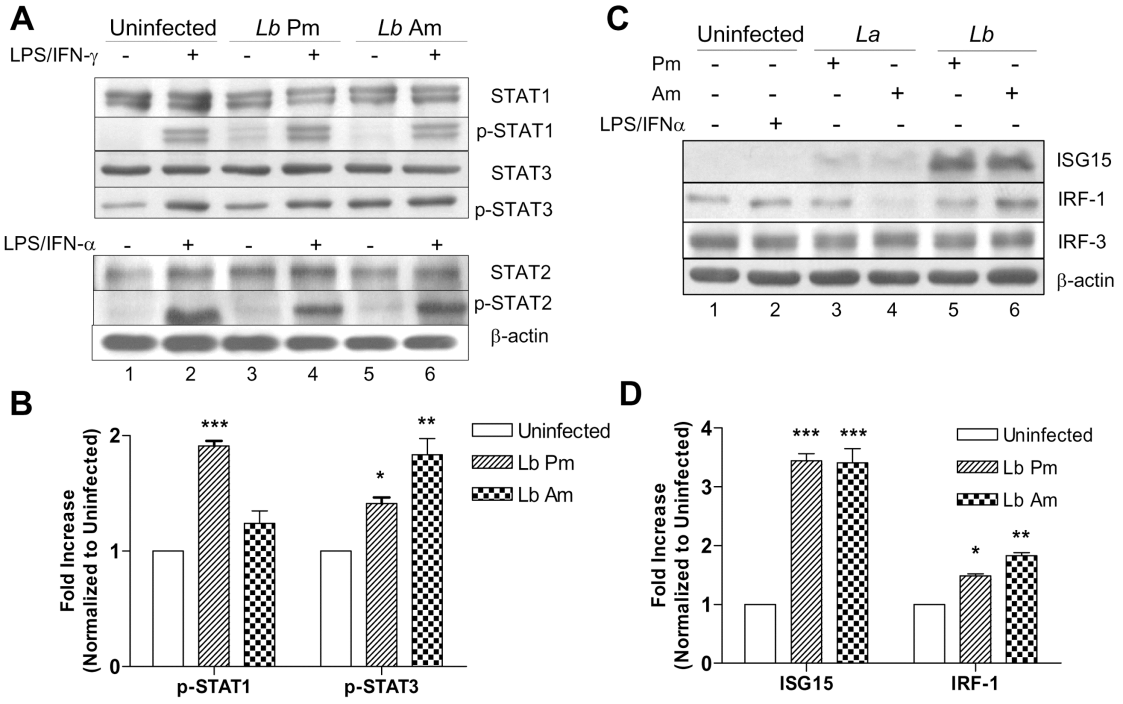

Figure 2.5 *L. braziliensis* infection up-regulates phosphorylated STAT molecules and ISG15. BMDCs were infected with *Lb* or *La* Pm or Am at 10:1 parasite-to-cell ratio for 6 h. Cells were then treated with (A) LPS/IFN-γ and LPS/IFN-α, or (C) LPS/IFN-α for 30 min. Expression levels of indicated total or phosphorylated proteins were evaluated using cell lysates on Western blots. Fold of increase for p-STAT1, p-STAT3 (B), and ISG15, IRF-1 (D) was calculated by densitometry analysis. Shown are representative images of three independent experiments with similar results. *(p<0.05), **(p<0.01) and ***(p<0.001), following one-way ANOVA analysis, indicate statistically significant differences between the control and infected groups.

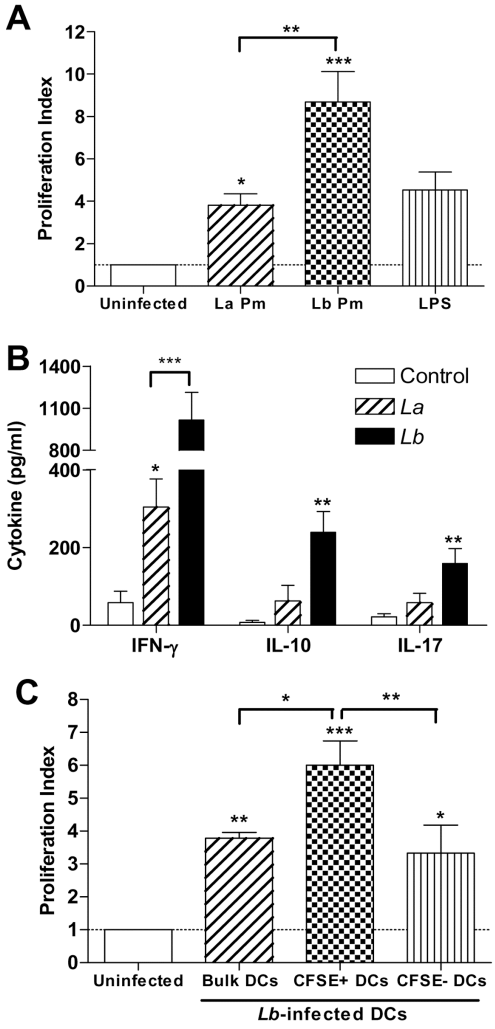

Figure 2.6 *L. braziliensis*-infected DCs are highly potent in priming naïve CD4$^+$ T cells *in vitro* and promoting the production of IFN-γ. BMDCs were infected with *Lb* and *La* Pm (A and B) and CFSE-labeled *Lb* Pm (C) at a parasite-to-cell ratio of 8:1 for 24 h. DCs were treated with mitomycin C (50 mg/ml) and co-cultured with spleen-derived naïve B6 CD4$^+$ T cells (2 x 10^6/ml) at an 1:10 DC-to-T ratio. LPS-treated DCs were used as a control. (A) T cell proliferation was measured by ^{3}H-thymidine uptake after 4 days of co-culture. (B) The presence of IFN-γ, IL-10 and IL-17 in culture supernatants was assayed by ELISA. (C) Enrichment of CFSE$^+$ (parasite-carrying) cells was performed by passing the cells through a FACSaria cell sorter. CFSE$^+$ and CFSE$^-$ DCs were then treated with mitomycin C prior to co-culture with naïve CD4$^+$ T cells. Shown are pooled data from three independent experiments. Bars represent statistically significant differences between compared groups. *(p<0.05), **(p<0.01) and ***(p<0.001) indicate statistically significant differences between the control and infected groups by one-way ANOVA analysis.

L. braziliensis*-infected DCs are highly efficient in priming and activating naïve CD4$^+$ T cells *in vitro

To examine the APC function of *Lb*-infected DCs, we used an *in vitro* T cell priming system, in which DCs were infected with parasites for 24 h, washed, and treated with mitomycin C prior to co-culture with spleen-derived naïve CD4$^+$ T cells. While DCs infected with *La* promastigotes stimulated T cell proliferation to some extent (p<0.05), DCs infected with *Lb* promastigotes were significantly more efficient in stimulating T cell proliferation (Fig. 2.6A) and T cell cytokine production (IFN-γ, IL-10, and IL-17) than *La* controls (p<0.001, Fig. 2.6B). Overall, *Lb*-infected DCs preferentially induced the generation of IFN-γ-, IL-10-, and IL-17-producing CD4$^+$ T cells, which was in sharp contrast to the low levels of cytokine production induced by *La*-infected DCs. To further evaluate the role of parasite-carrying DCs in T cell priming, we sorted CFSE$^+$ and CFSE$^-$ DC subsets and used them in T cell priming. Interestingly, we found that parasite-carrying, CFSE$^+$ DCs were highly efficient in stimulating T cell proliferation, and that bystander (CFSE$^-$) DCs were also capable of priming T cell proliferation to some extent (Fig. 2.6C). Collectively, these data suggest the relative contributions of parasite-carrying and bystander DCs in priming naïve CD4$^+$ T cells *in vitro*, and the potential of *Lb*-infected DCs in activating CD4$^+$ T cells to produce high levels of IFN-γ, IL-10 and IL-17.

Self-healing in *L. braziliensis*-infected mice is associated with an early induction and tight regulation of IFN-γ-producing $CD4^+$ T cells

To validate our *in vitro* T cell-priming observations, we next analyzed the $CD4^+$ T cell phenotype during *Lb* infection *in vivo*. B6 mice were infected s.c. with 2×10^6 *Lb* or *La* promastigotes in the hind foot, and lesion development was monitored weekly. As expected, *La* and *Lb* infections led to both non- and self-healing diseases, respectively (Fig. 2.7A), which correlated well with tissue parasite burdens at 4 and 8 weeks p.i. (Fig. 2.7B). To evaluate the phenotypes of *in vivo*-primed $CD4^+$ T cells, we examined the percentages (data not shown) and the total number of $CD4^+$ T cells in draining LNs. At 4 weeks, when comparable lesion sizes and parasite burdens were detected in both infection groups, there was a remarkable expansion (180×10^3 cells/LN) of IFN-γ-producing $CD4^+$ T cells in the LNs of *Lb*-infected mice ($p<0.001$, Fig. 2.7C). The percentages and total numbers of IFN-γ-, IL-17- and IL-10-producing $CD4^+$ T cells in *Lb*-infected mice, however, decreased considerably by 8 weeks (Fig. 2.7D), which correlated with parasite clearance (Fig. 2.7B). This early activation of IFN-γ-producing $CD4^+$ T cells was blunted in *La*-infected mice, but these mice displayed relatively high numbers of IFN-γ- and IL-10-producing cells at 8 weeks ($p<0.001$ and $p<0.01$). Similar statistical trends were obtained when LN cells were restimulated with soluble leishmanial Ags for 4 days (data not shown). These data collectively suggest that a strong induction of and fine balance among proinflammatory and regulatory cytokines is linked to the control of *Lb* infection.

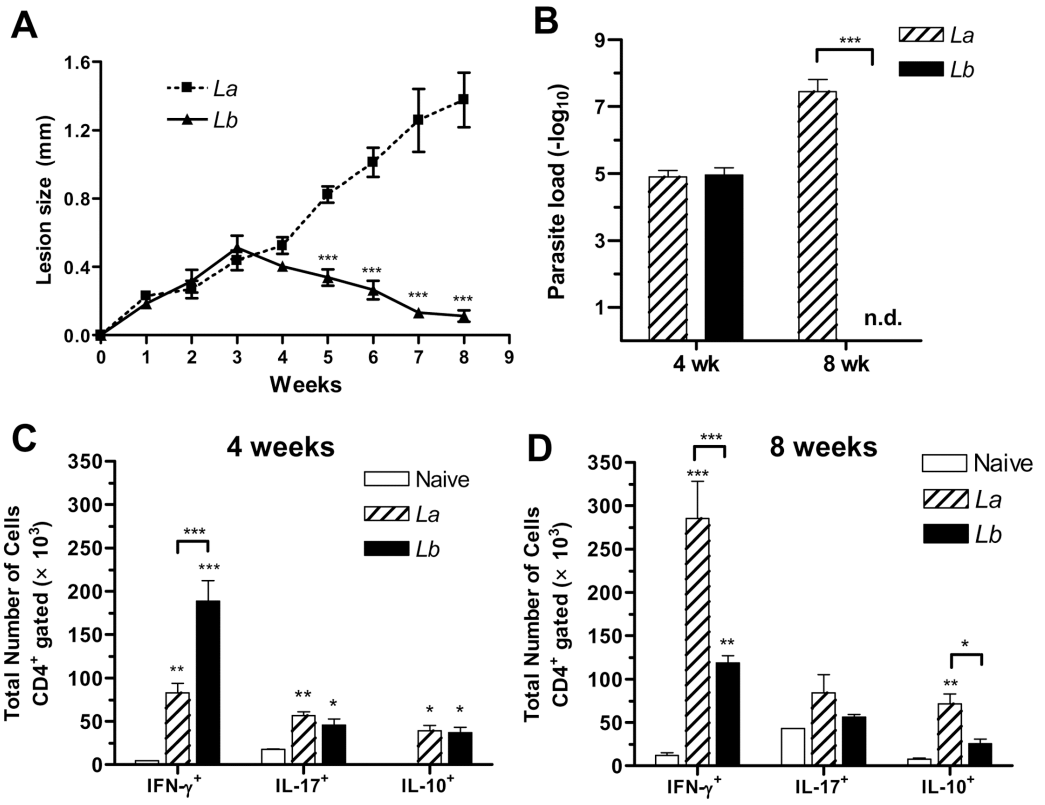

Figure 2.7 Self-healing in *L. braziliensis*-infected mice is associated with the expansion of IFN-γ-producing CD4$^+$ T cells. B6 mice (5/group) were infected in the hind foot with 2×10^6 metacyclic promastigotes of *Lb* or *La*. (A) Lesion sizes were monitored weekly with a digital caliper. (B) Mice were sacrificed at 4 and 8 weeks p.i., and parasite burdens in foot tissues were determined by limited dilution. Draining LN cells were collected at 4 (C) and 8 weeks p.i. (D) and stimulated with PMA/ionomycin/Golgi Plug for 6 h. Intracellular staining for IFN-γ, IL-17 and IL-10 was gated on CD4$^+$ T cells. Bars represent statistically significant differences between the compared groups. *($p<0.05$), **($p<0.01$) and ***($p<0.001$) indicate statistically significant differences between the naive and infected groups by one-way ANOVA analysis.

DISCUSSION

Recent studies from our laboratory, as well as that of others, have described how *La* infection in DCs and MΦs leads to a profound impairment in the IL-12 signaling pathway and alters the antigen-presenting capacity of these cells (49, 95, 101, 107, 108). However, little is known about how other New World *Leishmania* species interact with DCs at early stages of infection and how these interactions influence disease outcome. In this study, we examined in detail whether DCs responded to *Lb* pro- and amastigotes differently, as well as the downstream events triggered by these infections. We have provided evidence that, in sharp contrast to *La*, *Lb* (especially its amastigote form) can efficiently activate DCs to produce proinflammatory cytokines. To the best of our knowledge, this is the first report to describe DC-*Lb* interactions at the cellular and molecular levels. More importantly, this study provides new insight into early and differential DC responses to different *Leishmania* species and developmental stages and the impact of these responses on T cell activation and disease outcomes.

DC surface activation markers have been widely used to evaluate DC responsiveness to different pathogens. In this study, we used the surface expression of CD40, CD80, and CD83 molecules, as well as the production of IL-12p40, as the readouts for DC activation because of their known sensitivity to *Leishmania* infection *in vivo* (49, 51). In addition, we used CFSE-labeled pro- and amastigotes to define the status of DC activation at the single-cell level. Our *in vitro* DC infection and T cell-priming

studies have revealed several interesting findings. First, in contrast to *La* infection, which induces relatively low levels of DC maturation/activation (51), *Lb* infection induced high levels of activation in both parasite-carrying ($CD11c^+CFSE^+$) and bystander ($CD11c^+CFSE^-$) DCs (Fig. 2.3). While the "bystander activation" would be caused by a small number (less than 2%) of unlabeled parasites, it was more likely that it was due to cytokine/chemokine release from parasite-infected DCs. Second, the presence of *Lb* parasites did not alter DC responsiveness to secondary stimuli, because parasite-carrying DCs responded to LPS/IFN-γ by up-regulating surface activation markers and intracellular IL-12p40. Third, although both parasite-carrying and bystander DCs were capable of priming naïve $CD4^+$ T cells *in vitro*, the former were highly competent in doing so (Fig. 2.6). Therefore, strong DC activation is a hallmark for *Lb* infection.

In our *in vitro* studies, secreted and intracellular IL-12p40 proteins were readily detected in DCs infected with *Lb* pro- or amastigotes in the absence of other exogenous stimuli (Figs. 1-3). Similar to other reports (50, 96, 109), infection with parasites alone failed to stimulate IL-12p70 production, although *Lb*-infected, LPS/IFN-γ-stimulated DCs were capable of producing IL-12p70 (data not shown). Given that the expression of the IL-12p40 gene can precede the formation and release of bioactive IL-12p70, serving as an early event in host-pathogen interactions (110), we examined the expression of IL-12p40 and IL-12p35 genes via quantitative real-time RT-PCR. We found that infection with *Lb* pro- or amastigotes alone triggered a 45- to 55-fold increase of IL-12p40 mRNA at 8 h, which dropped dramatically by 24 h (Fig. 2.4), suggesting a transient induction

feature of the IL-12p40 gene during *Leishmania* infection (50). In our hands, *Lb* infection alone was insufficient to stimulate IL-12p35 gene expression in DCs (data not shown), which may explain the lack of detectable IL-12p70 in our in vitro studies. Given that production of IL-12p70 by APCs requires T cell-dependent signals, such as CD40-CD40L interactions (111), it will be interesting to further examine whether *Lb*-infected DCs are capable of producing IL-12p70 *in vivo* or in the presence of T cells *in vitro*.

The JAK/STAT signaling pathway not only plays a critical role in innate immunity by inducing expression of proinflammatory cytokines and anti-parasitic molecules (e.g., NO and oxygen radicals) (98, 112), but also affects cell fate by controlling the expression of genes involved in cell proliferation, differentiation and apoptosis (113). Therefore, it is not surprising that infection with *L. donovani* and *L. mexicana* promastigotes in Mϕs (98, 109) or *La* amastigotes in DCs (50) can down-regulate IL-12 production by altering the JAK/STAT and MAP kinase signaling pathways. Interestingly, we found that infection with *Lb* pro- and amastigotes enhanced the expression of p-STAT1 and p-STAT3 (Fig. 2.5B). Although the role of these phosphorylated proteins in *Lb* infection remains unclear, a recent report has suggested the necessity for STAT1 signaling on APCs, but not on T cells, in generating *L. major*-specific Th1 T cells and protective immunity (114). It appears that STAT3 has complex and multiple functions, serving as a positive regulator for DC homeostasis (115, 116), but as a negative regulator (through IL-10 transcription) for DC activation (117), because inhibition of the JAK2/STAT3 signaling can increase DC activation, maturation and the

capacity to induce T cell proliferation (118). Additional studies are needed to examine whether the up-regulation of p-STAT1 and p-STAT3 in *Lb*-infected DCs is responsible for the balanced Th1 immune response observed in infected animals (Fig. 2.7).

One of the novel findings in this study is the marked induction of ISG15 expression during *Lb*, but not *La*, infection (Figs. 2.4B and 2.5C). Although ISG15 is known to be important for immune responses against influenza, herpes and sindbis viruses (105), there are no reports of its involvement in parasitic infection. ISG15 is an ubiquitin-like molecule involved in a process known as ISGylation, which modifies the function of target proteins (119). Even though more than 100 proteins have been described as targets for ISGylation, the cellular functions of ISG15 and the modified proteins remain unknown (120). ISG15 can act within the immune system either by direct conjugation with target proteins [i.e. STAT1 (121)] or being secreted as a cytokine (105). The expression of ISG15 can be induced in different events, such as microbial infections, genotoxic stress, as well as pregnancy and retinoid-induced cellular differentiation (119). In an attempt to understand the biological relevance of ISG15 in *Leishmania* infection, we evaluated the levels of IRF-1 and IRF-3, two up-stream molecules for ISG15 (122). We detected an increase in IRF-1 proteins after *Lb* infection (Fig. 2.5C); however, no up-regulation of IRF-3 was detected, even though induction of IRF-3 is known to be correlated with up-regulation of ISG15 (123). Given that up-regulation of ISG15 appears to be selective for *Lb* infection, we are currently examining the biological role of the ISG15-related pathway using targeted gene knock-out mice (106).

Regardless of the mechanisms underlying a JAK/STAT- or ISG15-mediated intracellular event, there is no doubt that DC activation status can greatly influence Ag-specific T cell responses and, therefore, disease outcomes (124). Our *in vitro*, T cell-priming studies using two New World *Leishmania* species indicate clearly a correlation between DC activation status and the magnitude of T cell activation, confirming the high potential of *Lb*-infected DCs in priming naïve $CD4^+$ T cells (Fig. 2.6) and the generation of protective immunity in mice (Fig. 2.7). It is evident that hallmark of infection in *Lb*-infected mice is a rapid induction of IFN-γ-, IL-10-, and IL-17-producing $CD4^+$ T cells at early stages of infection, but a withdrawal of T cell responses during parasite clearance; however, such regulated responses are altered during *La* infection. Delayed and insufficient host responses in *La*-infected mice failed to control this infection, or may even promote amastigote growth intracellularly (54). While the roles of IFN-γ and IL-10 in murine models of leishmaniasis are well documented (43, 53, 125, 126), the role of IL-17 in CL is less clear. Our group has previously described a possible role for IL-17 during the immune response to *La* (51), especially because of the local tissue inflammation and neutrophil/MΦ infiltration observed during *Leishmania* infection (127). Given that IL-17-producing $CD4^+$ T cells are important for host defense against extracellular pathogens and organ-specific autoimmunity (128), and that IL-17 can induce the production of IL-1β, TNF and IL-6 in a variety of cell types (129), it will be interesting to define the role of the IL-17 network in CL and ML.

In summary, this study describes a signature feature of DC responses to *Lb* parasites. These detailed studies of DC responses to two New World species of *Leishmania* and their developmental stages provide new insights into how DCs differentiate invading parasites and how the responses at the molecular level impact on the development of protective and non-protective immune responses. Since *La* is the etiological agent for diffuse CL, which is a rare and immunosuppressive form of the disease (15), our comparative studies of DC infection with *Lb* vs. *La* and *La* vs. *L. major* (51) support the view that initial events at the DC level determine the timing and magnitude of *Leishmania*-specific $CD4^+$ T cell responses and the outcome of the infection. Given the clinical importance of ML and diffuse CL, it will be important to extend and confirm these findings in the human system. A better understanding of molecular details in DC-parasite interactions would provide novel strategies for the control of leishmaniasis.

CHAPTER 3: DISTINCT ROLES FOR MYD88 AND TLR2 DURING *LEISHMANIA BRAZILIENSIS* INFECTION IN MICE[2]

INTRODUCTION

Leishmaniasis is a vector-borne disease that has a great socio-economic impact in many tropical and neotropical countries (15). *Leishmania* parasites multiply as flagellated promastigotes in the midgut of sand flies and are transmitted to the vertebrate host via the bites of parasite-carrying female flies (14, 130). The insult at the bite site initiates a strong neutrophil influx and parasite capture by these cells (13). Interestingly, some of the captured parasites remain viable, and these infected neutrophils actually facilitate the silent entry of parasites into MΦs (131), where parasites survive and replicate as intracellular amastigotes (14). The magnitude and nature of inflammatory responses at the infection site and the profile of subsequent T cell responses determines the outcome of the infection. In South America, *Lb* infection causes CL in most cases and ML in some individuals. The latter is a severe and disfiguring form of the disease. At present, it remains unclear why the infection is controlled in some individuals but progressive in others (15).

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DC-pathogen interactions are initiated by interaction between receptors on DCs and pathogen-associated molecular patterns (PAMPs), including LPS, glycolipids, and nucleic acids. Signals through TLRs can induce DC maturation and the production of proinflammatory cytokines (74, 132), thereby bridging the innate and adaptive immune responses (133). Upon ligand binding, downstream signaling of all TLRs (with the exception of TLR3) uses the adaptor protein MyD88 (64). Gene knockout studies in mice have suggested that TLR signaling is essential for the immune responses against *Leishmania* parasites (134). For example, MyD88 and TLR4 contribute to the control of *L. major* infection in C57BL/6 mice (45, 135). TLR9 is involved in NK cell activation in animal models of visceral (*L. donivani*) and CL (*L. major* and *Lb*) (136, 137), while TLR2 and TLR3 are required for the intracellular killing of *L. donovani* in IFN-γ-primed MΦs (73). *Leishmania* lypophosphoglycan (LPG), an abundant molecule in the surface of promastigotes, is not only a virulence factor for some *Leishmania* species (e.g., *L. major* and *L. donovani*) (138), but also acts as a ligand for TLR2-mediated signaling (72). However, different species of *Leishmania* display relatively high variations (biochemical modifications) in LPG molecules (139). In the case of *Lb*, the procyclic form of the parasite lacks side-chain sugar substitutions on its LPG, whereas the metacyclic form appears to contain decreased amounts of LPG when compared to other *Leishmania* species (140). On the DC surface, TLR2 is present as pre-existing heterodimers of TLR2/1 and/or TLR2/6, recognizing triacylated and diacylated lipoproteins, respectively (67). TLR2 has been shown to be important for NK cell activation *in vitro* by purified *L.*

major LPG (72); however the functional roles of TLR2 remain largely unclear during both parasite-DC interactions and the course of *Leishmania* infection *in vivo.*

Most inbred strains of mice are genetically resistant to *Lb* infection, due to the capacity of mice to establish a strong Th1 response (141). This self-control of infection is accompanied by the selective expansion of IFN-γ-producing $CD4^+$ T cells, which induce nitric oxide production in infected MΦs to promote parasite killing (14, 43). We have previously revealed that several key molecules in the innate immunity pathways (e.g., STAT1, STAT3, and ISG15) were up-regulated in *Lb*-infected DCs, and that such DCs were highly efficient in priming $CD4^+$ T cells *in vitro* and *in vivo* (142). However, it remains unclear whether DC-*Leishmania* interactions in the absence of MyD88 and TLR2 impacts T cell functions and *in vivo* containment of infection. In the present study, we generated BMDCs from $MyD88^{-/-}$ and $TLR2^{-/-}$ mice and examined their responsiveness to *Lb* infection. We found that infected $MyD88^{-/-}$ DCs showed low levels of cell activation and IL-12p40 production, which correlated with an increased susceptibility of these mice to *Lb* infection and a decreased expansion of IFN-γ- and IL-17-producing $CD4^+$ T cells during the course of infection. Given that most TLR pathways share MyD88, and that TLR2 is involved in LPG recognition, we then examined the role of TLR2 in *Lb* recognition. Contrary to $MyD88^{-/-}$ DCs, the lack of TLR2 enhanced DC activation, IL-12p40 production, and T cell priming *in vitro*. Consequently, $TLR2^{-/-}$ mice were more resistant to infection than were control mice, a finding that was associated with enhanced IFN-γ production in the draining lymph nodes (dLN). Collectively, our

results show that while MyD88 is critical for *Lb* recognition *in vitro* and *in vivo*, and that TLR2 appears to have a regulatory role in modulating immune responses to the parasite.

MATERIALS AND METHODS

Mice: Female TLR2$^{-/-}$ mice (9 generations back-crossed to B6) and WT B6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME). MyD88$^{-/-}$ mice (10 generations back-crossed to B6) were bred at Baylor College of Medicine, Houston, as described previously (143). Mice were maintained under specific pathogen-free conditions and used at 6 to 8 weeks (wk) of age, according to protocols approved by the Institutional Animal Care and Use Committees.

Parasite culture and Ag preparation: Infectivity of *Lb* (LTB111) was maintained by regular passage through golden Syrian hamsters (Harlan Sprague Dawley, Indianapolis, IN). Infectivity of *La* (LV78) was maintained by regular passage through BALB/c mice (Harlan Sprague Dawley). Promastigotes were cultured at 23°C in Schneider's *Drosophila* medium (Invitrogen, Carlsbad, CA), pH 7.0, supplemented with 20% FBS (Sigma, St. Louis, MO), 2 mM L-glutamine, and 50 μg/ml gentamicin. Stationary promastigote cultures of less than five passages were used for DC or animal infection. To prepare soluble leishmanial Ags (SLA), *Lb* promastigotes (2×10^8/ml) were subjected to

six freeze-and-thaw cycles in liquid nitrogen and a 15-min sonication. SLA preparations were aliquot and stored at -20°C until use.

DC generation and infection: BMDCs were generated from B6, MyD88$^{-/-}$ and TLR2$^{-/-}$ mice in complete IMDM (Invitrogen) containing 10% FBS, supplemented with 20 ng/ml recombinant GM-CSF (eBioscience, San Diego, CA), and harvested at day 8. Parasites (5×10^7/ml in PBS) were washed twice and incubated with DCs (8:1 or 10:1 parasite-to-cell ratio) at 33°C for 8 h, and then at 37°C for another 16 h. LPS (100 ng/ml) of *Salmonella enterica* serovar Typhimurium (Sigma) and IFN-γ (100 ng/ml, Leinco Technologies, St. Louis, MO) were used as positive controls in all of the experiments. Pam_3CSK_4 (100 ng/ml, InvivoGen, San Diego, CA), a synthetic agonist of TLR2, was used as a means of quality control in the TLR2$^{-/-}$ *in vitro* experiments. At 24 h p.i., culture supernatants were harvested for cytokine detection and cells were collected for FACS analysis.

T cell priming *in vitro*: Naïve CD4$^+$ T cells were purified from the spleens of B6 mice by negative selection using magnetic beads (Miltenyi Biotec, Alburn, CA), and their purity was routinely around 95%, as judged by CD4 staining and FACS analysis. Purified CD4$^+$ T cells (2×10^5) were co-cultured with parasite-infected, mitomycin C-pretreated DCs at a 10:1 ratio in 96-well plates for 4 days. Supernatants were harvested for cytokine detection. To assess T cell proliferation, we added 1 μCi of [^{3}H]thymidine 18 h before harvest, and determined incorporated radioactivity on a microplate scintillation and luminescence counter (Packard Instrument Company, Downers Grove, IL). Stimulation

indexes were calculated by normalizing proliferation of $CD4^+$ T cells induced by parasite-infected DCs to proliferation induced by uninfected DCs.

Intracellular staining and FACS analysis: The following specific mAbs and their corresponding isotype controls were purchased from eBiosciences: FITC-conjugated anti-IFN-γ (XMG1.2) and rat IgG2a; PE-conjugated anti-IL-10 (JES5-16E3); anti-IL-12/IL-23p40 (C17.8); anti-CD40 (1C10); anti-CD80 (16-10A1); and anti-CD83 (Michel-17); as well as rat IgG1, IgG2a, and IgG2b; PE-Cy5-conjugated anti-CD11c (N418); and hamster IgG; PerCP-Cy5.5 anti-IL-17A (eBio17B7) and rat IgG2a; APC-conjugated anti-CD4 (GK1.5) and rat IgG2b; and PE-Cy7-conjugated anti-CD8a (53-6.7) and rat IgG2a. APC-Cy7-conjugated anti-CD3e (145-2C11) and Armenian hamster IgG1 were purchased from BD Biosciences (Franklin Lakes, NJ). Briefly, cells were washed, blocked with 1 μg/ml FcRγ blocker (CD16/32, eBioscience), stained for specific surface molecules, fixed/permeabilized with a Cytofix/Cytoperm Kit (BD Biosciences), and then stained for specific intracellular molecules. To detect intracellular cytokines, we added 1 μl/ml of Golgi Stop (BD Biosciences) for the last 6 h of cultivation. Cells were read on a FACSCanto Flow Cytometer (BD Biosciences) and analyzed using FlowJo V8.5 Software (TreeStar, Ashland, OR).

Cytokine ELISA: The levels of cytokines in culture supernatants were measured by using ELISA kits purchased from BD Biosciences (IL-12p40 and IL-10) or eBioscience

(IFN-γ and IL-17A). Detection limits were 15 pg/ml for IFN-γ, 4 pg/ml for IL-10, 10 pg/ml for IL-12p40, and 8 pg/ml for IL-17, respectively.

SOCS1 and SOCS3 real-time RT-PCR: At 4 h p.i, total RNA was extracted from 1 ~ 2 x 10^6 *Leishmania*-infected DCs (10:1 parasite-to-cell ratio) using the RNeasy system (QIAGEN, Valencia, CA). Genomic DNA was digested with the on-column RNase-free DNase (QIAGEN). For detecting SOCS1 and SOCS3 transcripts, cDNA was synthesized from 2 μg of total RNA by using the Superscript III First-Strand System (Invitrogen) primed with random hexamers. Real-time RT-PCR was performed at the institutional Real-Time PCR core facility, and all reagents were purchased from Applied Biosystems (Foster City, CA) and consisted of a 20× assay mixture of primers; TaqMan MGB probes (FAM dye-labeled) for mouse SOCS1 and SOCS3 (P/N 4331182); pre-developed 18S rRNA (VIC dye-labeled, as an endogenous control); and TaqMan assay reagent (P/N 4319413E). Separate-tube (singleplex), real-time PCR was performed with 40 ng cDNA for both target genes and the endogenous control, by using Taqman Gene expression master mix (P/N 4370074). The PCR cycling parameters were: Uracil-N-glycosylase (UNG) activation at 50°C for 2 min; AmpliTaq activation at 95°C for 10 min; denaturation at 95°C for 15 sec; and annealing/extension at 60°C for 1 min (repeated 40 times) on ABI7000 (Applied Biosystems). Duplicate CT values were analyzed in Microsoft Excel by using the comparative CT(ΔΔCT) method, as described by the manufacturer (Applied Biosystems). The amount of target ($2^{-\Delta\Delta CT}$) was normalized to

endogenous reference (18s) and fold induction was calculated relative to uninfected control samples.

***In vivo* evaluation of infection and T cell activation:** *Lb* stationary promastigotes were injected subcutaneously (s.c.) in the right hind foot (2×10^6 parasites/mouse, 4 mice/group). Lesion sizes were monitored weekly with a digital caliper (Control Company, Friendswood, TX), and tissue parasite burdens were measured via a limiting dilution assay by using LDA software (Oxford University Press, New York, NY) (101). At 4 and 8 wk p.i., mice were euthanized, and the popliteal LN cells (1×10^6/well/ml) were collected and stimulated with PMA/Ionomycin/Golgi Plug for 6 h (*ex vivo*) prior to examination of intracellular IFN-γ, IL-17 and IL-10 expression. Similarly, LN cells were also cultured in the presence of SLA (1:1 parasite-to-cell ratio) for 72 h (recall response), and cytokine concentrations were assayed in culture supernatants by ELISA.

Statistical analysis: One-way ANOVA was used for multiple group comparisons. Differences between individual treatment groups were determined using a Student's t-test. A p value of ≤ 0.05 was considered statistically significant (GraphPad Software v4.0, San Diego, CA).

RESULTS

MyD88$^{-/-}$ DCs have impaired activation and cytokine production after *L. braziliensis* infection

MyD88 is essential for DC activation/maturation after *L. donovani* and *L. major* infection (45, 144, 145). Given that *Lb* infection in B6 mice is self-healing due to a strong Th1 immune response (142), we investigated whether MyD88 is required for parasite control. We generated BMDCs from WT and MyD88$^{-/-}$ mice and infected cells with *Lb* promastigotes at an 8:1 parasite-to-cell ratio. We consistently observed significantly lower percentages of activated DCs ($CD11c^{+}CD40^{+}$), when compared to those from the WT controls (Fig. 3.1A). Since marked differences are reported for DC responsiveness to *Leishmania* species (51, 142), we also infected MyD88$^{-/-}$ DCs with *La*, a closely related New World species known for its selective impairment in DC activation, and used them as controls.

Upon pathogen capture, cytokines produced by DCs can shape both innate and adaptive immune responses, and the production of IL-12p40 greatly promotes Th1 cell differentiation (56). As shown in Fig. 3.1B, *Lb*-infected MyD88$^{-/-}$ DCs produced significantly lower amounts of IL-12p40 than did WT counterparts ($p<0.01$). This

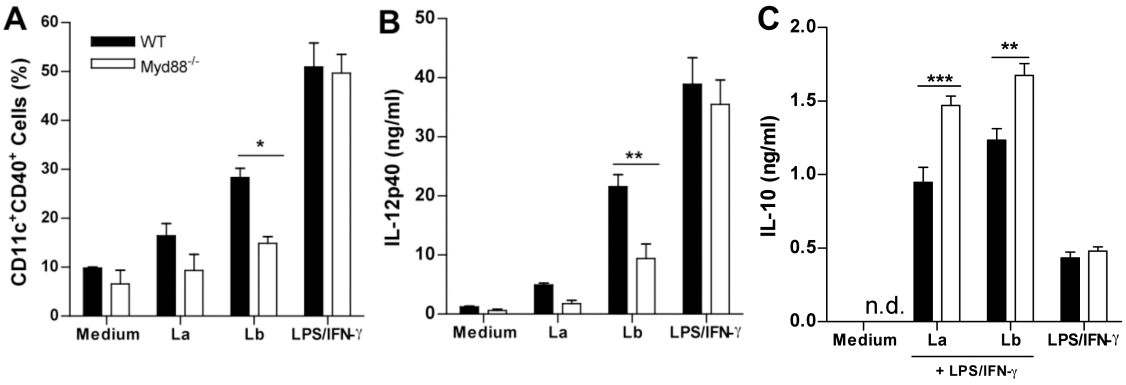

Figure 3.1 The absence of MyD88 impairs IL-12p40 production and DC activation after *L. braziliensis* infection. BMDCs were derived from WT (open bars) and MyD88$^{-/-}$ mice (closed bars) and infected with *Lb* and *La* promastigotes at an 8:1 parasite-to-cell ratio for 24 h. (A) Expression of CD40 on DC surface was measured by FACS. (B) IL-12p40 production in the culture supernatants was assayed by ELISA. (C) IL-10 production in the culture supernatants in response to parasite infection plus LPS (100 ng/ml) and IFN-γ (100 ng/ml) was assayed by ELISA. Results from three independent repeats were pooled. *($p<0.05$), **($p<0.01$), and ***($p<0.001$) indicate statistically significant differences between the groups by one-way ANOVA and Student's *t*-test analysis.

impairment was not due to an intrinsic failure in the knockout cells, since WT and MyD88$^{-/-}$ DCs produced similar levels of IL-12p40 following stimulation with LPS/IFN-γ (Fig. 3.1B). As compared to the WT counterparts, MyD88$^{-/-}$ DCs produced significantly higher amounts of IL-10 after infection with either *La* or *Lb* in the presence of LPS/IFN-γ (Fig. 3.1C). Thus, in the absence of MyD88, DC activation and IL-12p40 production after *Lb* infection is not only hampered, but also skewed towards an enhanced IL-10 production.

Enhanced susceptibility of MyD88$^{-/-}$ mice to *L. braziliensis* infection

It has been shown that MyD88$^{-/-}$ B6 mice are highly susceptible to *L. major* infection due to the skew from protective Th1 responses to a detrimental Th2-type immune response (45, 144, 145). To examine the role of MyD88 in the control of *Lb*, we infected MyD88$^{-/-}$ mice with 2×10^6 stationary promastigotes and monitored lesion development weekly. At 4 wk p.i., lesion sizes were comparable in both groups of mice; however, at 5 to 8 wk, a significant increase in lesion sizes was observed in *Lb*-infected MyD88$^{-/-}$ mice (Fig. 3.2A, $p<0.05$). Consistent with increased lesion sizes, infected MyD88$^{-/-}$ mice also contained significantly higher numbers of parasites in footpads at 8 wk, when compared to those of WT controls ($-\log_{10}$ 2.82 ± 0.35 vs. non-detectable, $p<0.001$). We followed up lesion progression for up to 12 wk p.i. and found that lesion size and parasite loads remained significantly higher in MyD88$^{-/-}$, when compared to those of WT controls (data not shown). To understand the mechanisms underlying the increased susceptibility in MyD88$^{-/-}$ mice, we examined the cytokine profiles of *in vivo* primed CD4$^+$ T cells in the dLN. At 4 wk p.i., when comparable lesion sizes (Fig. 3.2A) and parasite burdens (data not shown) were detected in both groups, MyD88$^{-/-}$ mice had significantly lower numbers of IFN-γ- and IL-17-producing CD4$^+$ T cells (Fig. 3.2B). The frequencies of cytokine-producing CD4$^+$ T cells remained significantly low in infected MyD88$^{-/-}$ mice even at 8 wk, by which time WT mice had already cleared the parasites (Fig. 3.2C). We observed similar trends when dLN cells were re-stimulated with parasite antigens for 3 days (data not shown). Our results suggest that MyD88 is

necessary for the generation of immune responses against *Lb* infection *in vivo*, especially for the priming of IFN-γ- and IL-17-producing $CD4^+$ T cells.

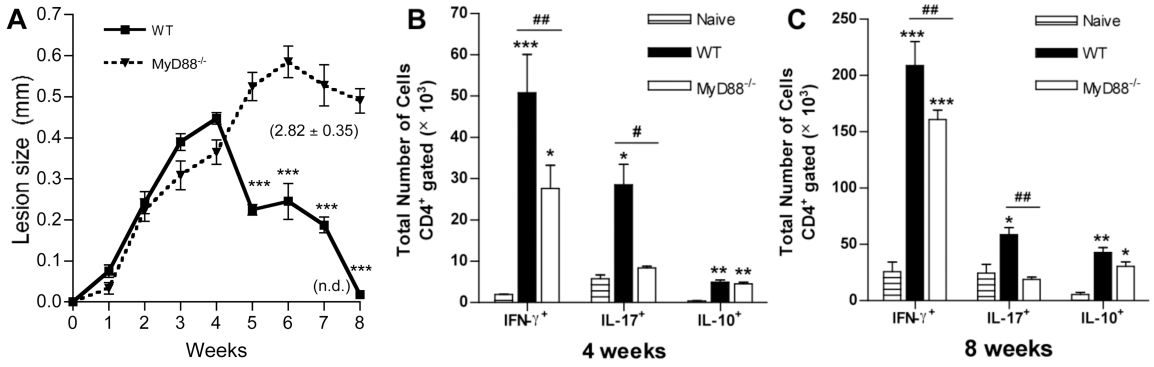

Figure 3.2 Increased susceptibility of MyD88$^{-/-}$ mice to *L. braziliensis* infection is associated with a decrease in IFN-γ- and IL-17-producing $CD4^+$ T cells. MyD88$^{-/-}$ and WT mice (4/group) were infected in the hind foot with 2×10^6 promastigotes of *Lb*. (A) Lesion sizes were monitored weekly with a digital caliper. Parasite loads at 8 weeks p.i. are indicated inside the graph ($-\log_{10} \pm$ SD), n.d., not detectable. At 4 (B), and 8 (C) weeks, dLN cells were collected and stimulated with PMA/ionomycin/Golgi Plug for 6 h. Intracellular staining for IFN-γ, IL-17 and IL-10 was gated on $CD4^+$ T cells. Results shown are from one experiment and representative of two independent experiments. $^{\#}$ ($p<0.05$), and $^{\#\#}$ ($p<0.01$) represent statistically significant differences between WT and MyD88$^{-/-}$ mice. *($p<0.05$), **($p<0.01$) and ***($p<0.001$) indicate statistically significant differences between the naive and infected groups by one-way ANOVA and Student's *t*-test analysis.

L. braziliensis-infected TLR2$^{-/-}$ DCs display high levels of activation and APC function

Given that MyD88 signaling is shared by almost all TLRs, and that TLR2 is a key molecule in host-*Leishmania* interactions (73, 146), we then investigated if TLR2 was

involved in the recognition of *Lb*. We generated DCs from TRL2$^{-/-}$ mice and infected them with *Lb* promastigotes. Unexpectedly, we found that the absence of TLR2 resulted in an increased expression of CD40 (Fig. 3.3A) and CD80 (Fig. 3.3B) in CD11c^{+} cells. Upon *Lb* infection, TLR2$^{-/-}$ DCs produced higher amounts of IL-12p40 (Fig. 3.3C), but lower amounts of IL-10 (Fig. 3.3D), than did WT DCs. It is known that TLR engagement can induce regulatory pathways, including the expression of suppressor of cytokine signaling (SOCS) molecules (147), and that *Leishmania* infection can activate the SOCS1 and SOCS3 genes in human MΦs (148). Therefore, we investigated whether changes in the transcription of the SOCS genes could explain the enhanced activation in infected TLR2$^{-/-}$ DCs. To our surprise, *Lb*-infected TLR2$^{-/-}$ DCs had significantly higher levels of SOCS1 (Fig. 3.3E) and SOCS3 transcription (Fig. 3.3F) when compared to infected WT DCs, suggesting a TLR2-independent up-regulation of SOCS1 and SOCS3 transcription in *Lb*-infected DCs.

The finding of enhanced DC activation following *Lb* infection in the absence of TLR2 prompted us to examine DC-T cell interactions. Using an *in vitro* T-cell priming assay, we found that co-culture of *Lb*- or *La*-infected TLR2$^{-/-}$ DCs with purified naïve CD4^{+} T cells resulted in a significant increase in both T cell proliferation (Fig. 3.4A) and IFN-γ production (Fig. 3.4B). Collectively, our *in vitro* infection studies suggested to us that TLR2 deficiency promotes DC activation and IL-12p40 production, in addition to CD4^{+} T cell activation and IFN-γ production.

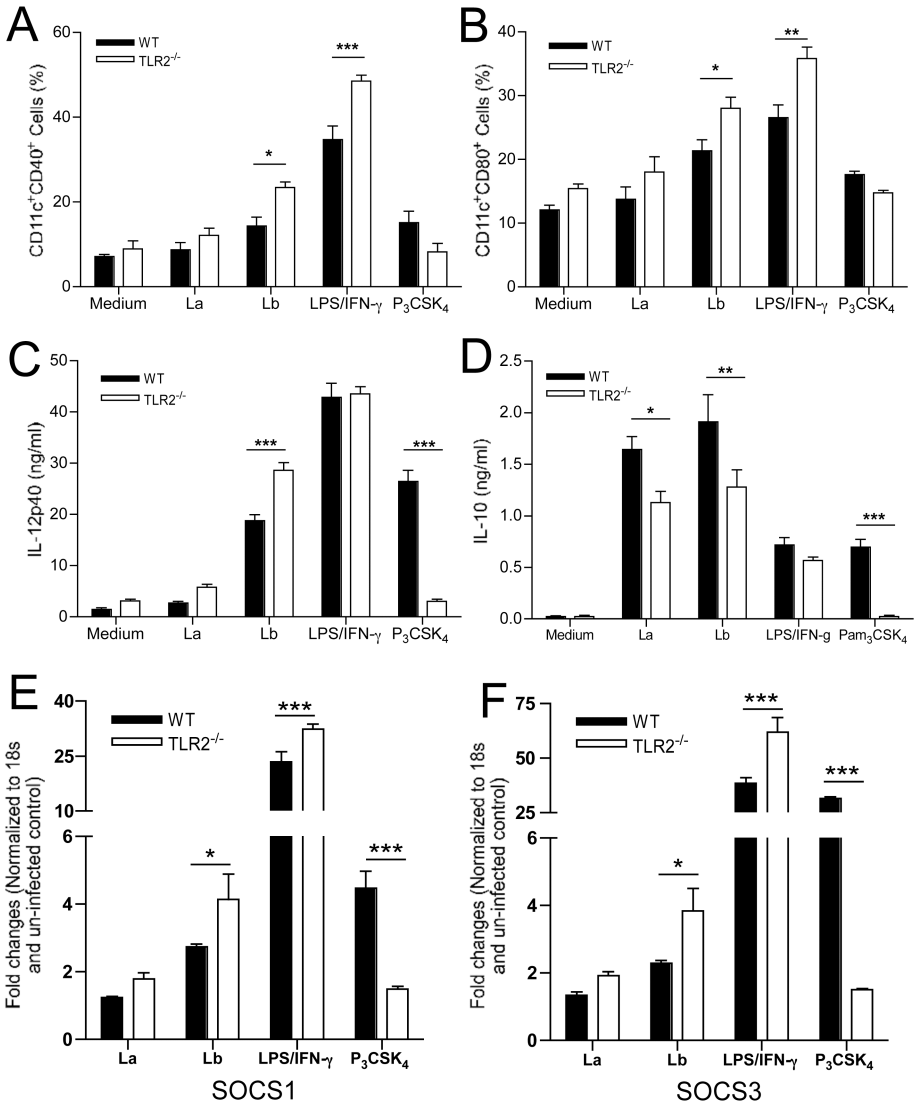

Figure 3.3 TLR2$^{-/-}$ DCs show an enhanced activation after *L. braziliensis* infection. WT and TLR2$^{-/-}$ BMDCs were infected with *Lb* and *La* promastigotes at an 8:1 parasite-to-cell ratio for 24 h. Expression of DC surface maturation markers CD40 (A), and CD80 (B) was measured by FACS. The levels of IL-12p40 (C) and IL-10 (D) in the culture supernatants were assayed by ELISA. (E-F) DCs were infected at a 10:1 parasite-to-cell ratio for 4 h before RNA extraction. Total RNA was extracted and used for real-time RT-PCR measurement of SOCS1 (E) and SOCS3 levels (F). Data are expressed as fold change of expression relative to un-infected samples and normalized to 18s ribosomal control. P_3CSK_4 (Pam_3CSK_4) was used as a quality control for TLR2. Data were pooled from three independent repeats and shown in the plots. *(p<0.05), **(p<0.01), and ***(p<0.001) indicate statistically significant differences between the groups by one-way ANOVA and Student's t-test analysis.

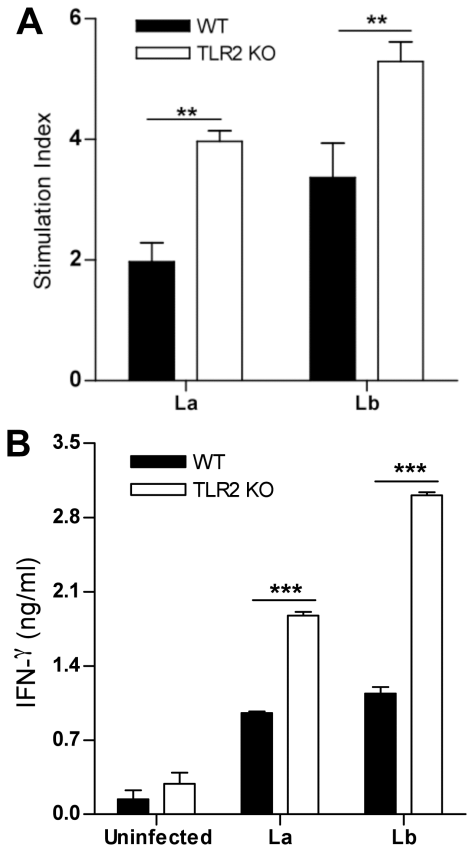

Figure 3.4 *Leishmania*-infected TLR2$^{-/-}$ DCs induce stronger *in vitro* T cell responses than do WT DCs. WT and TLR2$^{-/-}$ BMDCs were infected with *Lb* and *La* promastigotes at an 8:1 parasite-to-cell ratio for 24 h. DCs were then treated with mitomycin C (50 mg/ml) and co-cultured with spleen-derived, naïve syngeneic CD4^{+} T cells (2 x 10^{6}/ml) at a 1:10 DC-to-T cell ratio. (A) T cell proliferation was measured by ^{3}H-thymidine uptake after 4 days of co-culture. (B) The presence of IFN-γ in culture supernatants was assayed by ELISA. Shown are pooled data from three independent experiments. **(p<0.01) and ***(p<0.001) indicate statistically significant differences between the groups by one-way ANOVA and Student's *t*-test analysis.

Enhanced resistance of TLR2$^{-/-}$ mice to *L. braziliensis* infection *in vivo*

To examine the biological functions of TLR2, we infected WT and TLR2$^{-/-}$ mice with 2×10^6 promastigotes in the hind foot. We consistently observed that TLR2$^{-/-}$ mice developed significant smaller lesions, when compared to those found in infected WT mice from 2 to 5 wk p.i. (Fig. 3.5A), even though lesions from both groups contained comparable parasite loads at 4 and 8 wk (Fig. 3.5B). To determine if this enhanced protection in infected TLR2$^{-/-}$ mice was associated with changes in T cell cytokine profile, we assessed *in vivo* primed CD4$^+$ T cells via FACS. Both infection groups contained comparable numbers of IFN-γ-, IL-17- and IL-10-producing CD4$^+$ T cells at 4 wk (Fig. 3.5C); however, the numbers of IFN-γ-producing CD4$^+$ T cells in TLR2$^{-/-}$ mice were significantly higher than those in WT mice at 8 wk (p<0.01, Fig. 3.5D). By comparison, the numbers of IL-17- and IL-10-producing CD4$^+$ T cells were comparable in TLR2$^{-/-}$ and WT mice at 8 wk (Fig. 3.5D). To confirm the sustained production of IFN-γ in infected TLR2$^{-/-}$ mice, we re-stimulated dLN cells *in vitro* with *Lb* antigens for 3 days and measured IFN-γ and IL-10 levels in culture supernatants by ELISA. As shown in Fig. 3.6A, while Ag-specific IFN-γ production decreased markedly from 4 to 8 wk p.i. in WT mice (p<0.05), IFN-γ production was maintained at a relatively high level during this period in TLR2$^{-/-}$ mice. This trend was unique for IFN-γ since IL-10 production by dLN cells in response to antigen restimulation was reduced in both TLR2$^{-/-}$ and WT mice by 8 wk (Fig. 3.6B).

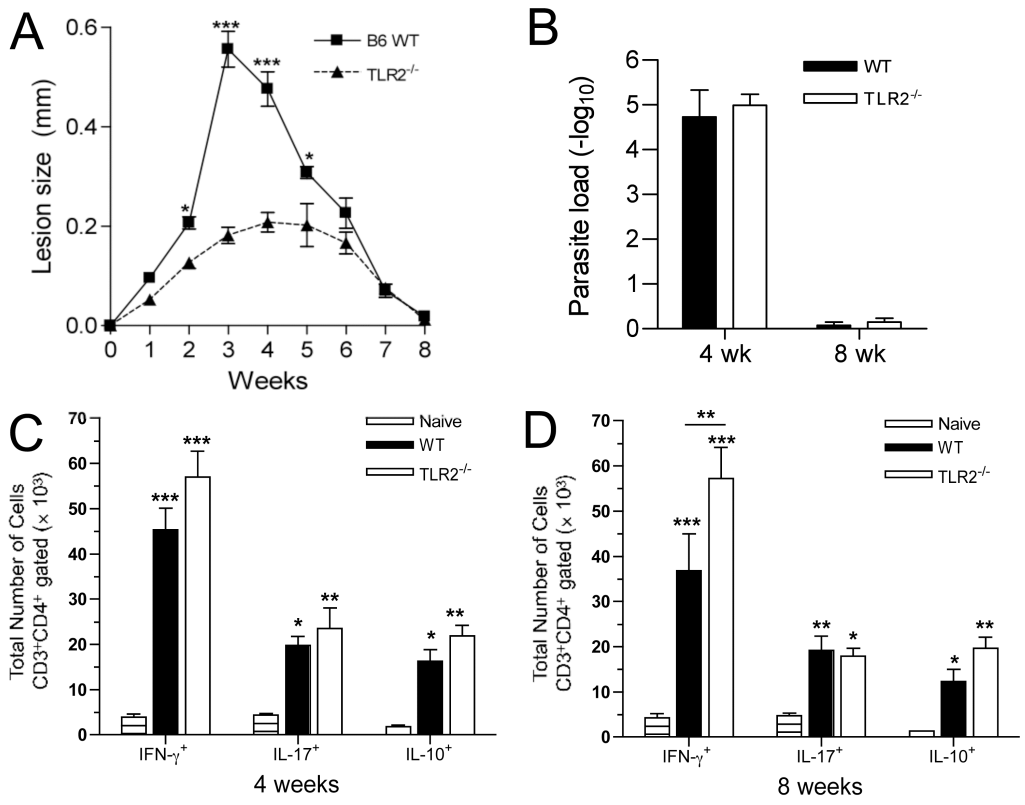

Figure 3.5 TLR2$^{-/-}$ mice have enhanced resistance to *L. braziliensis* infection. TLR2$^{-/-}$ and WT mice (5/group) were infected in the hind foot with 2 × 10^6 promastigotes of *Lb*. (A) Lesion sizes were monitored weekly with a digital caliper. (B) Mice were sacrificed at 4 and 8 wk, and parasite burdens in foot tissues were determined by limited dilution. Draining LN cells were collected at 4 (C) and 8 wk p.i. (D) and stimulated with PMA/ionomycin/Golgi Plug for 6 h. Intracellular staining for IFN-γ, IL-17 and IL-10 was gated on CD3$^+$CD4$^+$ T cells. $^{\#\#}$ (p<0.01) represents statistically significant differences between WT and TLR2$^{-/-}$ mice. *(p<0.05), **(p<0.01) and ***(p<0.001) indicate statistically significant differences between the naive and infected groups by one-way ANOVA and Student's *t*-test analysis.

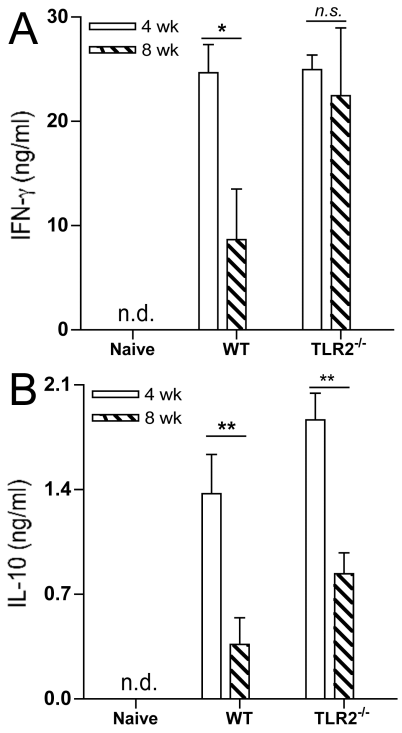

Figure 3.6 Decreased susceptibility of TLR2$^{-/-}$ mice to *L. braziliensis* infection is associated with a sustained production of IFN-γ in the draining lymph nodes. TLR2$^{-/-}$ and B6 WT mice (4/group) were infected in the hind foot with 2×10^6 promastigotes of *L. braziliensis*. At 4 and 8 weeks, draining LN cells were collected and re-stimulated with parasite antigens for 3 days. Supernatants were collected and assayed by ELISA to determine the concentrations of IFN-γ (A) and IL-10 (B). Shown are representative results from one of two independent experiments with similar trends. *($p<0.05$) and **($p<0.01$) indicate statistically significant differences between the groups by one-way ANOVA and Student's *t*-test analysis. N.d., not detectable.

DISCUSSION

Since their discovery, TLRs have been extensively studied for their role in the recognition of pathogen components and the initiation of innate immune responses (64, 149). We have recently described that *Lb* infection triggers DC activation and cytokine production, partially via the activation of the JAK/STAT signaling pathway (142). However, little is known about how innate immunity contributes to *Lb* recognition by DCs and parasite containment *in vivo*. In the present study, we used both an *in vitro* DC infection system and an *in vivo* infection model to analyze the roles of MyD88 and TLR2 in *Lb-DC* interactions and in the generation of antigen-specific $CD4^+$ T cell responses. Our data indicate that MyD88 was important for DC recognition of *Lb* promastigotes *in vitro* and for the control of infection *in vivo* because of significant impairments in DC and T cell responses and protective immunity in $MyD88^{-/-}$ mice. Unexpectedly, we found that DC and T cell activation in response to *Lb* infection *in vitro* was markedly enhanced in the absence of the TLR2 gene. As such, $TLR2^{-/-}$ mice were more resistant to *Lb* infection than were WT controls, partially due to the sustained production of IFN-γ in infected knockouts. To the best of our knowledge, this is the first report to describe the differential roles of MyD88 and TLR2 in *Lb*-DC interactions and the generation of protective immunity to this parasite species.

Our studies involving MyD88$^{-/-}$ DCs are in line with previous reports, confirming that in the absence of this adaptor protein, DC maturation and pathogen recognition are affected (45, 150-152). The first control point for the establishment of a protective Th1 immune response is at the level of IL-12 production by DCs, a process that can be regulated by MyD88-dependent and –independent mechanisms (153). In *Toxoplasma gondii* infection models, it has been shown that the production of both IL-12p40 and IL-12p70 in splenic DCs is mediated via MyD88-dependent events (154). In our hands, *Lb*-infected, but not *La*-infected, MyD88$^{-/-}$ DCs expressed lower amounts of IL-12p40 and co-stimulatory molecule CD40 than did WT cells (Fig. 3.1). It is documented that *La* parasites can directly affect antigen presentation by targeting the JAK/STAT signaling pathway in MΦs (99) and DCs (50), therefore down-regulating IL-12 production and Th1 responses. Thus, our comparative studies involving two distinct New World *Leishmania* species clearly indicate that successful DC activation by *Lb* parasites is partially dependent on the TLR-MyD88 signaling pathway. A second mechanism to regulate Th1 cell development by APCs is the induction of IL-10 production (51). Although infection of DCs with promastigotes alone did not trigger IL-10 production (142), we observed that infection with both parasite species, in the presence of LPS/IFN-γ, caused significantly higher levels of IL-10 secretion in MyD88$^{-/-}$ DCs than it did in their WT controls (Fig. 3.1C). Collectively, our *in vitro* results suggest that the absence of MyD88 differentially affects IL-12p40 and IL-10 production by *Leishmania*-infected DCs.

MyD88$^{-/-}$ mice have been reported to be more susceptible to infection with a wide variety of viral, bacterial and protozoan pathogens, such as Respiratory Syncytial Virus (155), *Burkholderia pseudomallei* (156), *T. gondii* (154), *Plasmodium berghei* (157) and *L. major* (45, 144, 145). Our *in vivo* studies with *Lb* also indicate that MyD88 is required for effective parasite clearance and lesion resolution in B6 mice, a mouse strain that spontaneously heals the lesions at around 8 wk (44). We have previously reported that *Lb* infection leads to a strong induction of IL-17-producing CD4^{+} T cells (Th17 cells) both *in vitro* and *in vivo* (142). Interestingly, *Lb* infection in MyD88$^{-/-}$ mice failed to trigger Th17 cell differentiation throughout the observation period (Fig. 3.2B-C). It is known that Th17 immune responses are crucial during infections that cause tissue inflammation and cell recruitment (158), especially because IL-17 can induce the production of IL-1β, TNF-α and IL-6 in different cell types (129). Studies in a murine model of inflammatory bowel disease showed that in the absence of MyD88, Th17 cell differentiation was strongly and particularly affected (159), a finding that was similar to those in our studies. Even though Th17 cells seem to play an important role during infection, Th1 activation is a prerequisite for the development of a protective immune response against *Leishmania* parasites (1). In agreement with this view, we observed that the lack of MyD88 caused a decrease, but not a complete abolishment, in the intensity of Th1 responses. Our results add up to previous reports showing a crucial role for MyD88 in the establishment of an IL-12-mediated Th1 immune response against *L. major* (45, 144, 145). MyD88-dependent resistance to *Lb* infection can also be attributed to TLR-independent pathways, because certain inflammatory responses (e.g., the IL-1 and IL-8 signaling pathways) also

use MyD88 as an adaptor protein (160). Since *Lb* is not extremely pathogenic in mice, it is not surprising that the infected MyD88$^{-/-}$ mice displayed an increased susceptibility with delayed parasite clearance, rather than uncontrolled parasite replication as observed in *L. major*-infected MyD88$^{-/-}$ mice (45, 145). Furthermore, we did not observe significant production of IL-4 by dLN cells of WT and MyD88$^{-/-}$ mice at 8 and 12 weeks p.i. (data not shown), a finding that was in contrast to *L. major*-infected MyD88$^{-/-}$ mice (45, 145). Since the IL-17-producing CD4^{+} T subset appeared to be mostly affected by the absence of MyD88, we are currently investigating the role of Th17 cells in *Leishmania* infection.

TLR2 can bind to a wide variety of pathogenic molecules, including lipoteichoid acid, peptidoglycan, atypical LPS and other lipoproteins from both Gram-positive and Gram-negative bacteria (161, 162). Importantly, the surface of *Leishmania* promastigotes is intensively covered by GPI-anchored and -related proteins (such as gp63 and LPG) (140, 163). De Veer *et al*. have described that purified *L. major* LPG can activate MAPK signaling and induce the production of IL-12p40 and TNF-α in bone marrow-derived MΦs, in a TLR2- and MyD88-dependent manner (144). Activation of TLR2 signaling, however, can also induce regulatory pathways in a wide variety of cell types (164). For example, findings from studies of human peripheral blood DCs stimulated with peptidoglycan and *Porphyromonas gingivalis* fimbriae showed a decreased capacity of the DCs to stimulate allogeneic T cell proliferation when compared to LPS-stimulated DCs (165). Similarly, Ropert *et al*. showed that splenocytes of TLR2$^{-/-}$ mice produce

increased levels of IFN-γ and nitric oxide after *in vivo* challenge with *T. cruzi*, when compared to wild-type controls (166). Our *in vitro* studies with DCs derived from TLR2$^{-/-}$ and WT mice showed that *Lb* infection induced significantly high levels of DC activation and IL-12p40 production (Fig. 3.3A-C), as well as decreased levels of IL-10 production (Fig. 3.3D). These results suggest a negative/regulatory role for TLR2 in DC activation. This conclusion was supported by our findings that, in the absence of TLR2, *Leishmania*-infected DCs were highly potent APCs for activating syngeneic CD4^{+} T cells to proliferate and produce IFN-γ (Fig. 3.4). It has been shown that *L. donovani* is capable of inducing expression of SOCS1 and SOCS3 in human MΦs as a potent inhibitory strategy to prevent cell activation (148). Our real-time RT-PCR studies for the expression levels of SOCS1 and SOCS3 suggest that up-regulation of these proteins in *Lb*-infected DCs (Fig. 3.3) is TLR2-independent. At this stage, the intracellular events that lead to the enhanced DC and T cell activation in *Leishmania*-infected TLR2$^{-/-}$ mice remain unclear.

Several infection models have indicated that TLR2 deficiency enhances the susceptibility of mice to bacterial and viral pathogens such as *Streptococcus pneumoniae* (167) and Herpes Simplex Virus (168). Regardless of the mechanisms underlying enhanced DC activation during *Lb* infection in the absence of TLR2, our *in vivo* studies indicate that TLR2$^{-/-}$ mice were capable of controlling *Lb* and developed smaller lesions than did the WT controls (Fig. 5A). Despite this, *Lb*-infected TLR2$^{-/-}$ and WT mice showed comparable parasite loads at 4 weeks p.i., suggesting that the reduction in lesion sizes in TLR2$^{-/-}$ mice was not linked to increased parasite killing in this model (Fig.

3.5B). The decreased lesion sizes in TLR2$^{-/-}$ mice correlated with an enhancement in IFN-γ production in LN cells in response to re-stimulation with *Lb* SLA, especially at late stages of the infection (Figs. 3.5 and 3.6). These *in vivo* and *ex vivo* findings clearly support our *in vitro* data, which suggested to us that enhanced DC activation and T cell priming capacity had a positive impact on protective immunity to *Leishmania* infection *in vivo*.

In summary, this study addresses the role of MyD88 during the immune response to *Lb* infection and how the absence of this molecule greatly impairs protective immunity. Additionally, we show that not all signaling pathways that use MyD88 act alike, since the absence of TLR2 had a positive impact on disease outcome. Findings from our *in vitro* studies using MyD88$^{-/-}$ and TLR2$^{-/-}$ DCs, which were similar to those in our previous reports (48), have revealed a direct correlation between DC activation status *in vitro* and the outcome of infection in the host. Furthermore, unraveling the role played by key components of the innate immune system would provide novel strategies for the control of leishmaniasis.

CHAPTER 4: CXCL10 PRODUCTION BY HUMAN MONOCYTES IN RESPONSE TO *LEISHMANIA BRAZILIENSIS* INFECTION[3]

INTRODUCTION

Leishmaniasis is a tropical disease caused by infection with protozoan parasites from the genus *Leishmania.* Worldwide, the disease affects 12 million people, with 350 million people currently considered at risk of infection (15). *Leishmania* parasites are transmitted by infected female sand flies, which inoculate the metacyclic promastigotes into the skin during a blood meal (130). Parasites are able to survive initial capture by neutrophils and later replicate or persist within MΦs, monocytes, DCs and fibroblasts as intracellular amastigotes (13, 87, 169). *Lb* is a New World *Leishmania* species that is highly endemic in many geographical areas of Central and South America (15, 170). *Lb*-infected individuals initially develop CL; however, in 5-10% of the patients, the disease further develops into ML (171). ML is a severe and disfiguring form of the disease, characterized by uncontrolled T and B cell responses to the parasite, and is caused almost exclusively by *Lb* infection (15). Currently, the host- and pathogen-associated factors that lead to ML progression are largely uncharacterized.

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Pathogen interactions with components of the host immune response are crucial for the establishment of protective immunity and pathological responses. Given that monocytes and MΦs serve as the main reservoir for *Leishmania* parasites during active infection (14), it is of interest to study the early events associated with parasite uptake. Uptake of *Leishmania* parasites can induce monocytes and MΦs to produce different chemokines, which in turn can skew immune responses by recruiting and inducing different components of the immune system (172). Chemokines are small (8-10 kDa) chemoattractant cytokines that play important roles during immune responses by triggering integrin activation, and inducing the recruitment of antigen-specific lymphocytes to peripheral tissues in response to inflammation (173). At present, approximately 50 human chemokines and 20 chemokine receptors have been characterized (174). CXCL10 (also known as the 10-kDa IFN-γ-inducible protein, IP-10) is a small molecule that is secreted mainly by monocytes, fibroblasts and endothelial cells in response to stimuli such as viral infection, LPS, IL-1β, and IFN-γ (175, 176). The biological function of CXCL10 involves the recruitment of monocytes, MΦs and T cells to sites of inflammation (175). Furthermore, it is known that during active leishmaniasis, lesion cells from self-healing CL patients produce Th1-mobilizing chemokines, such as CXCL10 and CXCL9 (172). Recruitment of IFN-γ-producing Th1 cells favors MΦ activation and parasite clearance; however, excessive production of these molecules could potentially lead to uncontrolled inflammation and tissue destruction (172, 177).

In the present study, we used an *in vitro* infection system to analyze the early events associated with *Lb* infection of human peripheral blood mononuclear cells (PBMCs). We found that *Lb* infection selectively induced the transcription of CXCL10, CCL2 (Monocyte chemotactic protein-1, MCP-1) and CCL4 (MΦ inflammatory protein-1-beta, MIP-1β). Furthermore, we showed that within PBMCs, *Leishmania* infected monocytes and upregulated expression of CXCR3 (G protein-coupled receptor 9, CD183) on the cell surface. Interestingly, only *Lb* infection induced the production of CXCL10, as PBMCs infected with *La* (another New World species of *Leishmania* that can cause immune suppression in patients) failed to do so. To further validate our results, we measured the presence of these inflammatory mediators in the sera of ATL patients from a *Lb* endemic region, and found that the levels of CXCL10, CCL2, CCL4 and sTNFRII (soluble TNF receptor type II) were significantly increased in both CL and ML patients. Collectively, our results indicate that immune responses against New World species of *Leishmania* are differentially regulated, and that the excessive production of inflammatory mediators could potentially exacerbate disease severity in *Lb*-infected individuals.

MATERIALS AND METHODS

Sample collection and preparation: Blood samples were collected by venipuncture of healthy volunteers into sodium heparin-coated collection tubes (BD Biosciences), and PBMCs were obtained by centrifugation on Accuprep gradients (Accurate Chemical). Cells were washed thoroughly and re-suspended at 1×10^6 cells/ml in RPMI media (Invitrogen), supplemented with 10% FBS (Hyclone), 1% non-essential aminoacids, 1 mM sodium piruvate, 50 μg/ml kanamycin, and 2 mM L-glutamine (all from Sigma). Human monocytes were obtained by positive selection using CD14 microbeads (Miltenyi Biotec). To differentiate MΦs, monocytes were cultured for 5 days in complete RPMI media supplemented with 20 ng/ml recombinant human GM-CSF (Peprotech). Serum samples from 27 ATL patients (13 CL and 14 ML) were collected after clinical evaluation and upon admittance into the Leishmaniasis Working Group at the Tropical Medicine Institute "Alexander von Humboldt" in Universidad Peruana Cayetano Heredia in Lima, Peru. Parasitological diagnosis (either by smear microscopy, PCR or parasite culture) was confirmed in 70% of CL and 65% of ML cases, respectively. Inflammatory mediators in serum were detected by using a Custom Quantibody Array (RayBiotec). Serum samples obtained from non-infected individuals (n = 13) were used as controls for the Quantibody Array. All cell and serum samples used in this study were collected after informed consent and approved by the Institutional Review Board at UTMB and the Ethics Committee at Universidad Peruana Cayetano Heredia.

Parasite culture: Infectivity of *Lb* (LTB111) and *La* (LTB0016) was maintained by regular passage through golden Syrian hamsters and BALB/c mice (Harlan Sprague Dawley), respectively. Promastigotes were cultured at 23°C in Schneider's *Drosophila* medium (Invitrogen), pH 7.0, supplemented with 20% FBS, 2 mM L-glutamine, and 50 μg/ml gentamicin (Sigma). Stationary promastigote cultures of fewer than five passages were used for cell infection.

Cell infection and transwell assays: PBMCs and MΦs were infected at a 5:1 parasite-to-cell ratio at 33°C for 4 h, and then at 37°C for another 20 h. LPS (100 ng/ml) of *Salmonella enterica* serovar Typhimurium (Sigma) and recombinant human IFN-γ (100 ng/ml, Leinco Technologies) were used as positive controls. At 24 h p.i., culture supernatants were harvested for cytokine detection, and cells were collected and stained for FACS analysis. For cell migration experiments, 5-μm transwell systems were used (24-well, Costar). Briefly 1×10^6 infected PBMCs, monocytes (5:1 parasite-to-cell ratio) were seeded in the basal chamber, and 1.5×10^6 non-infected, donor-matched, CFSE-labeled (2.5 μM, Sigma) PBMCs were seeded into the apical chambers. After incubation for 4 h at 33°C and then at 37°C for 10 h, cells in the basal chamber were collected, counted and stained for FACS analysis. Recombinant human CXCL10 (20 ng/ml, eBiosciences) was used as a positive control for cell migration.

Cytokine ELISA: The cytokine levels in culture supernatants were measured by using ELISA kits purchased from eBiosciences (IFN-γ and IL-10) or RayBiotec (CXCL10).

Detection limits were 4 pg/ml for IFN- γ, 2 pg/ml for IL-10, and 8 pg/ml for CXCL10, respectively.

Reverse-transcription PCR: At 4 h p.i., total RNA was extracted from 2×10^6 infected PBMCs (5:1 parasite-to-cell ratio) by using the RNeasy system (Qiagen), and genomic DNA was digested with the on-column RNase-free DNase (Qiagen). cDNA was synthesized from 2 μg of total RNA by using the Superscript III First-strand system (Invitrogen) primed with random hexamers. All PCR reactions were performed by using 2 μl of cDNA, the Platinum TaqDNA polymerase system and specific primer pairs. For CXCL10, CCL4 and β-actin, PCR reactions included 30 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 1 min, with a final 7-min extension step at 72°C. For CCL2 and CCL3, the same reaction conditions were used, but with 58°C as the annealing temperature. PCR products (8 μl) were subjected to electrophoresis on 1% agarose gels and visualized by staining with ethidium bromide. Results were quantified using the AlphaEase FC densitometry analysis software (v4.0, Alpha Inotec) and normalized to the expression of the β-actin gene and the non-stimulated controls.

Flow Cytometry: Unless specified, all human-specific mAbs and staining reagents were purchased from BD Biosciences: Alexa Fluor 488-conjugated anti-CXCR3 (1C6/CXCR3) and mouse IgG_{1k}, and PE-conjugated anti-CXCL10 (6D4/D6/G2) and mouse IgG_{2a}, The following reagents were purchased from eBioscience: APC-Cy7-

conjugated anti-CD3 (SK7) and mouse IgG_{1k}, PE-Cy5.5-conjugated anti-CD56 (MEM188) and mouse IgG_{2a}, APC-conjugated anti-IFN-γ (4S.B3) and mouse IgG_{1k}, and PE-Cy7-conjugated anti-CD14 (61D3) and mouse IgG_{1k}. Briefly, cells were washed, stained for specific surface molecules, fixed/permeabilized with a Cytofix/Cytoperm kit, and then stained for specific intracellular molecules. To detect intracellular cytokines, we added 1 µl/ml of Golgi Stop for the last 6 h of culture. Cells were read on a FACSCanto Flow Cytometer (BD Biosciences) and analyzed by using FlowJo V8.5 Software (TreeStar).

Statistical Analysis: Unless otherwise specified, all data shown in figures were pooled from the indicated number of repeats. Differences between individual treatment groups were determined by using a Student's *t*-test. A p value of ≤ 0.05 was considered statistically significant (Prism v4.0, GraphPad Software).

RESULTS

Early activation of chemokine genes following *L. braziliensis* infection

We have previously reported that *Lb* infection in mice leads to the development of a robust Th1 immune response, which includes the expansion of IFN-γ- and IL-17-producing $CD4^+$ T cells (142). Furthermore, *Lb*, but not *La*, infection in mice induced the expression of several chemokines and chemokine receptors such as CXCL10, CXCR3, CCL4, CCR1, 2, 3, 5 at three weeks of infection (Fig. 4.1). Given that *Lb* and *La* infections in mice represent self-healing and non-healing CL, respectively, these findings support the view that early events involved in parasite recognition have a significant impact on disease outcome (142, 178). Human infections caused by *Lb* are rarely self-healing, and disease in 5-10% of patients can further progress into ML (15). Although many reports have described the clinical characteristics of ML (15, 179), very few have focused on the initial interaction between human cells and *Lb* parasites. We addressed this issue by analyzing chemokine gene expression following infection of healthy volunteer PBMCs with *Lb* parasites for 4 h and found an increased transcription of CXCL10, CCL2, and CCL4 in response to *Lb* infection (Fig. 4.2). We also included control cells infected with *La*, another New World species known to cause immune suppression in patients, and confirmed the specific responses triggered by *Lb* infection. Since CXCL10 is an important chemokine for cell recruitment and Th1 induction (176),

and its transcription was observed only in *Lb*-infected PBMCs (Fig. 4.2B), we decided to investigate the potential role for CXCL10 during *Lb* infection. As shown in Fig. 4.3A, PBMC infected with *Lb* parasites secreted higher levels of CXCL10, IFN-γ and IL-10, than those induced by *La* infection ($p<0.05$). Similarly, human MΦs also produced higher levels of CXCL10 at 24 and 48 h of infection with *Lb* but not *La* parasites (Fig. 4.3B).

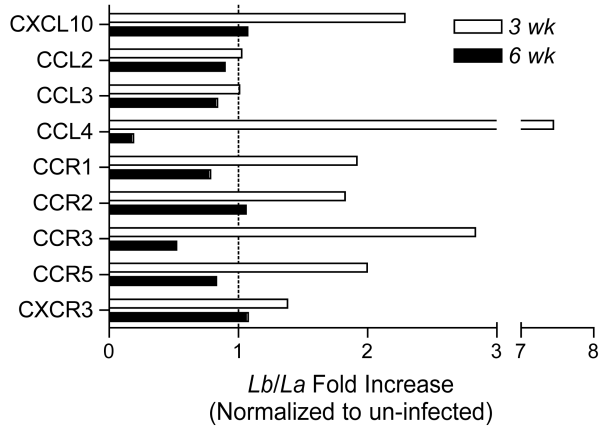

Figure 4.1 Early expression of proinflammatory mediators during *L. braziliensis* infection *in vivo*. Female B6 mice (n = 4) were infected in the ear dermis with stationary promastigotes of *Lb* or *La* (1×10^6). At 3 and 6 wk p.i., ear lesion tissues were collected and pooled for RNA extraction. The whole genome microarray analysis was performed by Miltenyi Biotec. The expression levels of indicated genes were normalized to un-infected ear tissues of age-matched mice. Results are shown as fold increases between the *Lb* and *La* groups at the indicated time points. The dotted line indicates comparable expression levels between the two infection groups.

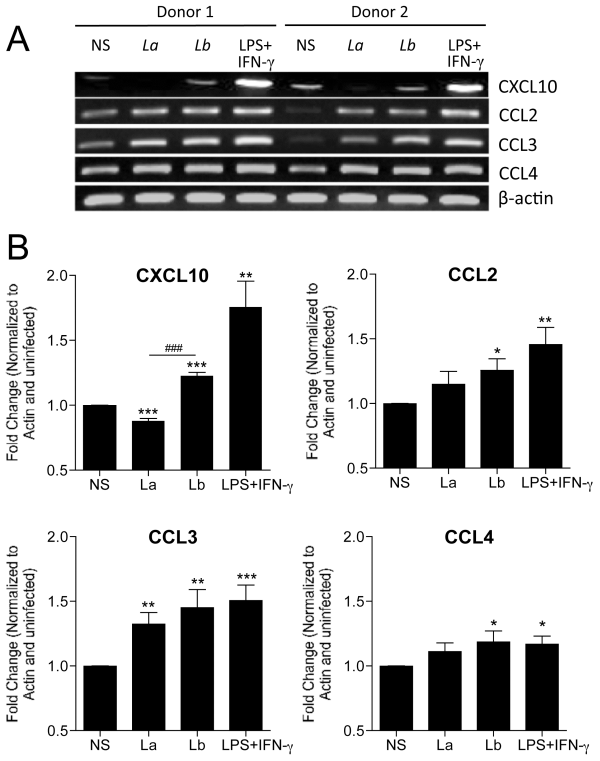

Figure 4.2 Activation of chemokine gene transcription by human PBMCs in response to *L. braziliensis* infection. Human PBMCs were isolated from healthy donors (n = 8) and infected with *Lb* or *La* promastigotes at a 5:1 parasite-to-cell ratio. At 4 h p.i, total RNA was extracted for RT-PCR analysis. (A) Gel images of CXCL10, CCL2, CCL3 (MΦ inflammatory protein-1-alpha, MIP-1α). and CCL4 transcripts from two donors. (B) Densitometry data obtained from all donors were pooled and shown in the plots. ### (p<0.001) represents statistically significant differences between the compared groups. *(p<0.05), **(p<0.01) and ***(p<0.001) indicate statistically significant differences by Student's *t*-Test between the non-stimulated controls and infected groups.

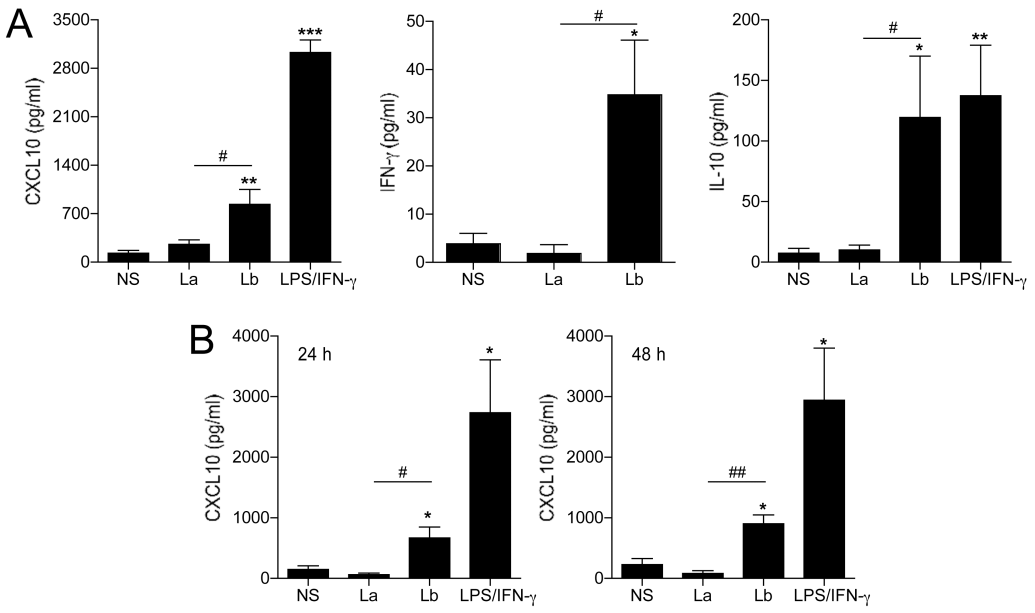

Figure 4.3 *L. braziliensis* infection induces CXCL10 production in human PBMCs and MΦs. (A) PBMCs (n = 10) were infected with *Lb* or *La* promastigotes at a 5:1 parasite-to-cell ratio for 24 h. (B) $CD14^{+}$ (n=3) monocytes were isolated and differentiated into MΦs and infected with promastigotes at a 10:1 parasite-to-cell ratio for 24 and 48 h. The level of indicated molecules in culture supernatants was assayed by ELISA. # ($p<0.05$) and ## ($p<0.01$) represent statistically significant differences between the compared groups. *($p<0.05$), **($p<0.01$) and ***($p<0.001$) indicate statistically significant differences by Student's *t*-Test between the non-stimulated controls and infected groups.

CXCL10 production and CXCR3 expression are induced by *L. braziliensis* infection

To determine the cell type susceptible to *Lb* infection and the origin of CXCL10 production, in the following experiments we used CFSE-labeled parasites. We observed that *Leishmania* infection was confined to $CD14^+$ cells within the monocyte gate because >95% of cells in the R_2 gate of infected groups were $CD14^+CFSE^+$ (Fig. 4.4B). Using LPS plus IFN-γ as a control, we observed that only $CD14^+$ cells (within the monocyte gate) produced CXCL10 (Fig. 4.4C). Consistent with ELISA results (Fig. 4.3A), our FACS studies confirmed that a marked CXCL10 production was only observed in response to *Lb* infection and by $CD14^+$ cells (Fig. 4.4D-E). Since cell responsiveness to CXCL10 chemotactic gradients depends on CXCR3 expression on the cell surface (176), we investigated whether parasite infection had any effect on surface expression of this receptor. We consistently observed that *Leishmania* infection up-regulated CXCR3 expression only on the surface of $CD14^+$ cells, but not on $CD14^-$ cells (Fig. 4.5A-B).

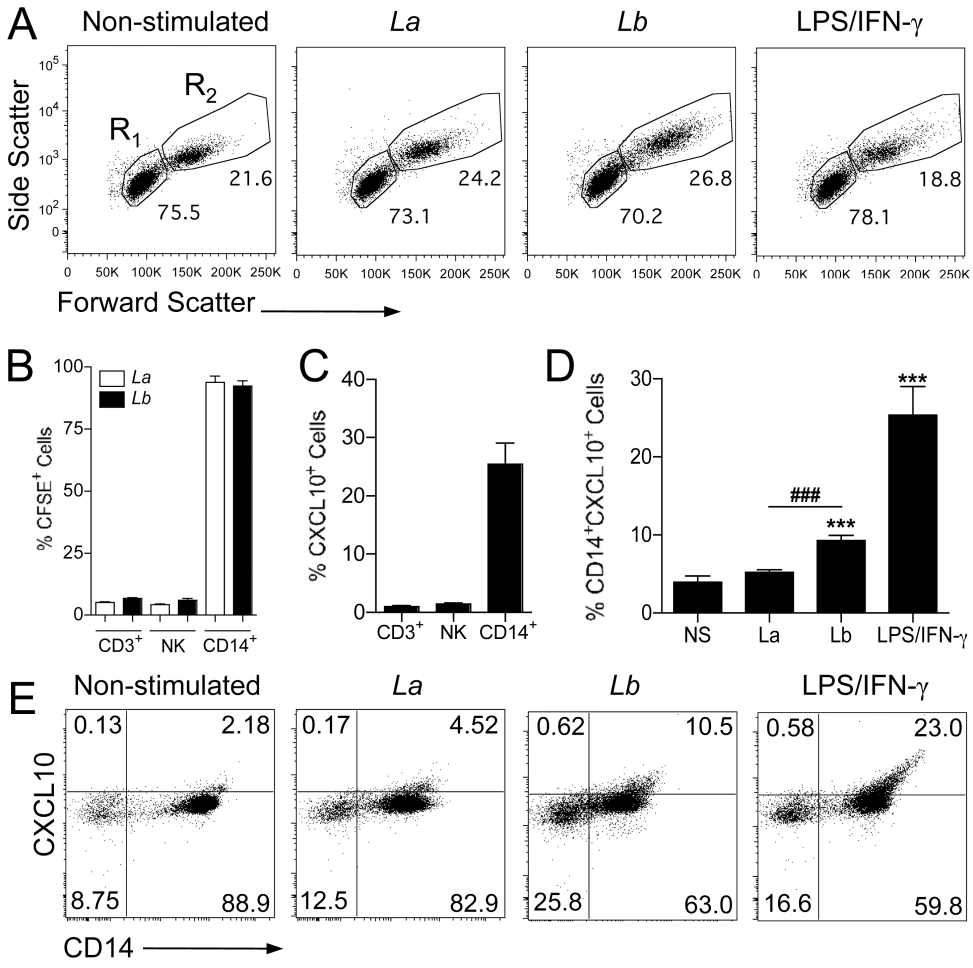

Figure 4.4 Monocytes are the major producers of CXCL10 following *L. braziliensis* infection. Human PBMCs were isolated from healthy donors (n = 6) and infected with CFSE-labeled *Lb* or *La* promastigotes at a 5:1 parasite-to-cell ratio for 24 h. (A) Representative forward vs. side scatter plots of PBMCs cultured under different conditions are shown. Gates used to analyze lymphocytes (R_1) and monocytes (R_2) are indicated. (B) CFSE intensity was used to determine the infection rates among T lymphocytes ($CD3^+$), NK cells ($CD3^-CD56^+$), and monocytes ($CD14^+$ cells within the monocyte gate). (C) CXCL10 production in different cell types in response to LPS+IFN-γ (100 ng/ml each) stimulation was assayed by FACS. (D-E) Intracellular CXCL10 in infected monocytes ($CD14^+CFSE^+$) was measured by FACS. ### ($p<0.001$) represents statistically significant differences between the compared groups. ***($p<0.001$) indicates statistically significant differences by Student's *t*-Test between the non-stimulated controls and infected groups.

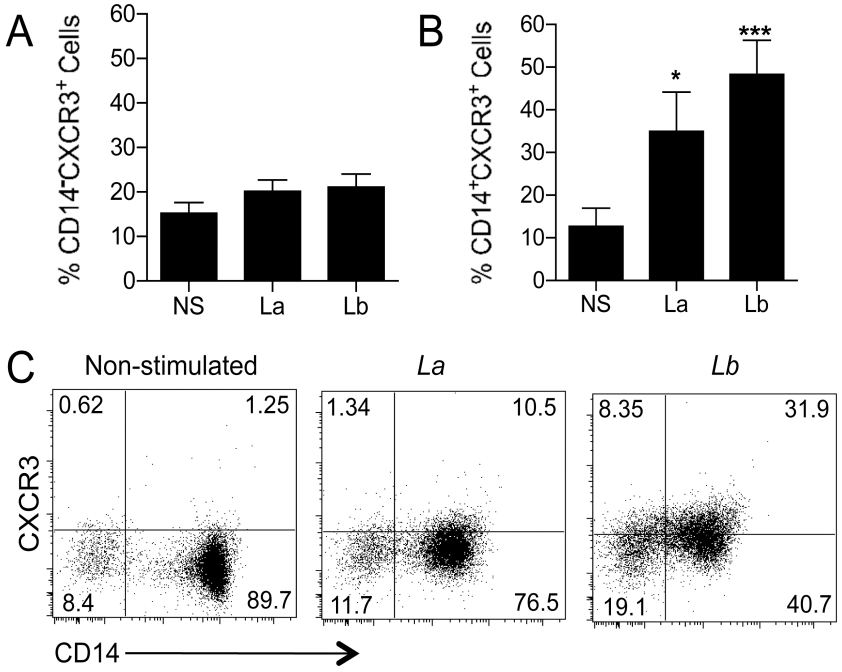

Figure 4.5 Monocyte-specific up-regulation of CXCR3 after *Leishmania* infection. PBMCs were isolated from healthy donors (n = 6) and infected with *Lb* or *La* promastigotes at a 5:1 parasite-to-cell ratio for 24 h. The levels of CXCR3 expression on $CD14^-$ (A) and $CD14^+$ cells (B-C) were measured by FACS. *($p<0.05$) and ***($p<0.001$) indicate statistically significant differences by Student's *t*-Test between the non-stimulated controls and infected groups.

L. braziliensis-infected cells efficiently recruit monocytes through chemotactic gradients

To investigate whether the increased production of chemokines following *Lb* infection had an impact on cell recruitment, we assessed the chemotactic properties of *Lb*-infected cells by using a 5-μm transwell system. Our results showed that both *Lb*-infected PBMCs and monocytes induced higher numbers of cell migration than did *La*-infected or non-infected cells (Fig. 4.6A). The use of CFSE-labeled PBMCs on the apical chamber allowed us to quantify and type the responding cells. As shown in Fig. 4.6B, chemoattractant factors released by *Lb*-infected cells induced the migration of higher numbers (~46.8%) of CFSE-labeled cells, when compared to those from *La*-infected (~34.3%) or non infected (~38.2%) cells. Further analysis of the migrated $CFSE^+$ cells revealed that *Lb*-infected cells preferentially induced the migration of $CD14^+$ monocytes (Fig. 4.6C, 61% as compared to 29.3% in the control), whereas soluble factors released after LPS/IFN-γ treatment preferentially induced the migration of $CD3^+$ T cells. These data suggest a selective recruitment of monocytes by *Lb*-infected cells.

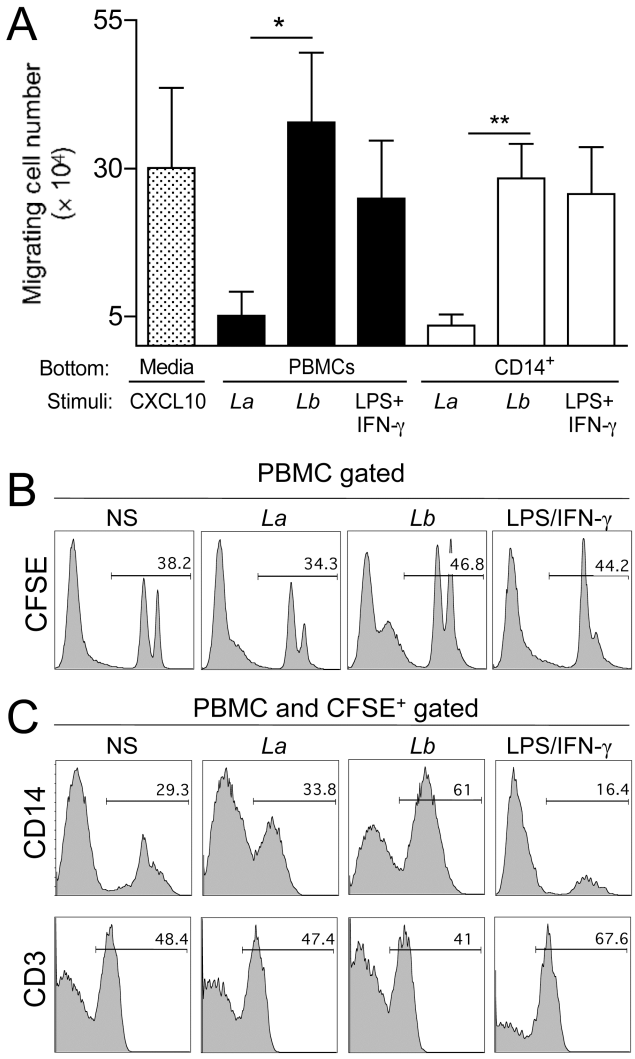

Figure 4.6 *L. braziliensis*-infected PBMCs and monocytes efficiently induce migration of non-infected PBMCs. PBMCs and $CD14^+$ cells were isolated (n = 4) and infected with *Lb* or *La* promastigotes at a 5:1 parasite-to-cell and placed in the basal chamber of 5-μm transwell plates. Non-infected, donor-matched PBMCs were labeled with CFSE and placed in the apical chamber and allowed to migrate in response to basal chamber released chemotactic factors for 14 h. (A) The total number of migrating cells present in the basal chamber was counted after the incubation period. Data was normalized by subtracting the migration observed in non-stimulated controls. (B) The percentage of migrating $CFSE^+$ PBMCs in the basal chamber was measured by FACS. (C) Percentages of monocytes ($CD14^+$) and T lymphocytes ($CD3^+$) within $CFSE^+$ migrating cells. (B-C) Histograms from one of four representative experiments are shown. ***(p<0.001) indicates statistically significant differences by Student's *t*-Test between compared groups.

Increased levels of inflammatory mediators in the serum of ATL patients

To validate and extend our *in vitro* observations, we analyzed the presence of inflammatory mediators in the serum of ATL patients (patient demographics described in Table 4.1). By using the RayBiotec Human Inflammation Antibody Array, we found in an initial screening of inflammatory factors high levels of CXCL10, CCL2, CCL4, CCL15, sTNFRI, sTNFRII and IL-18 in serum from CL and ML patients (data not shown). We then used a custom Quantibody Array and quantified the levels of 10 inflammatory factors in all of our samples. In accordance with our preliminary results, we detected significantly higher levels of CXCL10, CCL2, CCL4 and sTNFRII in the sera of CL and ML patients, when compared to those of control samples (Fig. 4.7). Our results suggest that responses to New World species of *Leishmania* are differentially regulated, and that the excessive production of inflammatory mediators during active leishmaniasis may have an adverse impact on disease outcome.

Parameter		Control (n = 13)	CL (n = 13)	ML (n = 14)
Gender (%)				
	Male	69%	69%	86%
	Female	31%	31%	14%
Age (years)				
	Mean ± SD	33 ± 9	41 ± 19	37 ± 10

Table 4.1 Demographic features of leishmaniasis cases and control subjects used in this study. Serum samples were obtained from patients after clinical confirmation of the disease but before the beginning of antimonial therapy. SD, standard deviation.

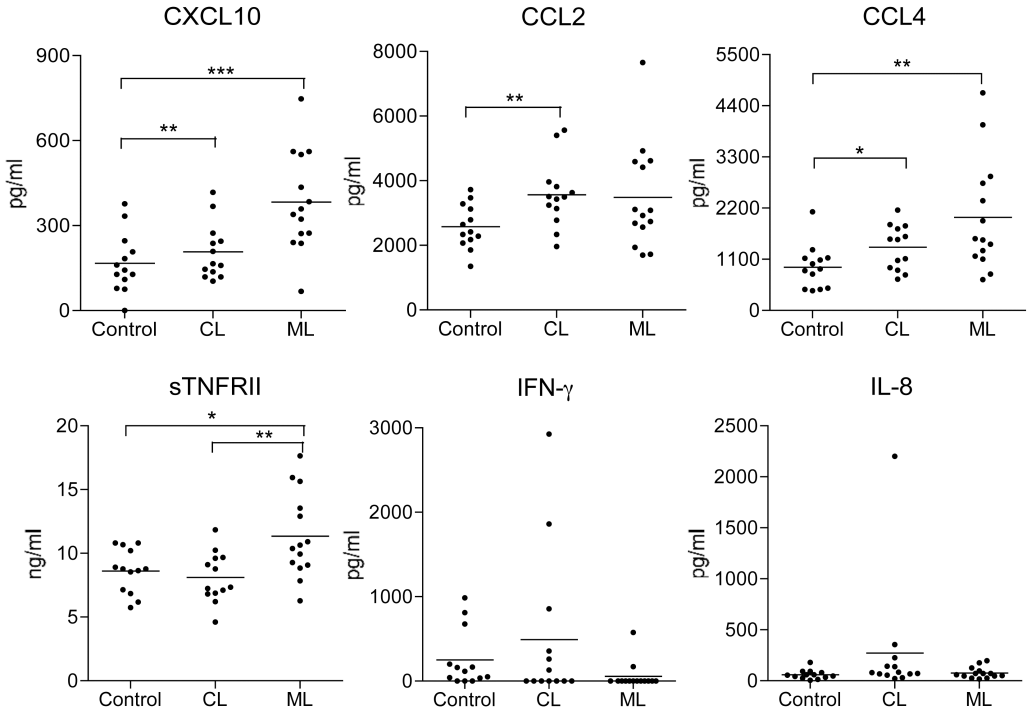

Figure 4.7 The profile of inflammatory chemokines and cytokines in the serum of American tegumentary leishmaniasis patients. Serum samples were collected from a total of 13 CL and 14 ML active patients. Samples from 13 healthy volunteers were used as controls. The levels of indicated molecules were measured with a RayBiotech ® Custom Quantibody Array. Bars represent statistically significant differences between the compared groups. *(p<0.05), **(p<0.01), and ***(p<0.001) indicate statistically significant differences by Student's t-Test.

DISCUSSION

Infection of healthy donors' PBMCs with *Leishmania* parasites can lead to induction or suppression of T cell proliferation, monocyte/DC maturation, and cytokine production, depending on the parasite species tested (87, 180-182). Most of these studies, however, focus on changes that occurred at or after 48 h of infection *in vitro*. During host-pathogen interactions, the initial production of chemokines and cytokines can influence both innate and adaptive immune responses (172). In this study, we investigated the early events following infection with two New World species of *Leishmania* parasites, paying special attention to the inflammatory mediators involved in cell recruitment. Our studies clearly indicate a rapid and robust induction of several chemokines/cytokines during *Lb,* but not *La*, infection in human cells *in vitro*, as well as in C57BL/6 mice [Fig. 4.1 and (142)]. Although the *in vivo* function of CXCL10/CXCR3 in human CL remains unclear at this stage, our findings of elevated serum levels of CXCL10 and other proinflammatory factors in patients with active skin and mucosal lesions suggest a possible involvement of these molecules in disease outcome.

Infection of human PBMCs with *Leishmania* parasites can trigger the expression of different sets of inflammatory chemokines. For example, *L. major*-treated PBMCs upregulated the transcription of CCL2 (183), whereas *L. donovani* infection triggers the production of TNF-α and IL-18 (184). In our hands, *Lb* infection preferentially induced a

strong expression of CXCL10 and CCL4, as judged by RT-PCR and ELISA (Figs. 4.2 and 4.3). A regulated production of these chemokines is required for pathogen clearance and wound healing due to their chemotactic potential in recruiting immune cells such as T cells, monocytes, DCs, neutrophils and NK cells (185-188). Of note, the CXCL10 transcription levels in *La*-infected cells were even lower than those observed in non-stimulated controls ($p<0.05$, Fig. 4.2). This finding was consistent with previous reports in human monocytes (87) and our studies in murine DCs and MΦs (50, 189), indicating a profound suppression of host innate immunity during *La* infection (82).

The role of CXCL10 in human leishmaniasis is not clear, although CXCL10 is well known for its involvement in recruiting monocytes, MΦs, T cells and NK cells (172) during human viral and bacterial infections (190, 191), and organ transplantation (192). By using a murine model of *La* infection, our group has previously shown that exogenous CXCL10 promotes parasite killing in MΦ cultures *in vitro* (52), enhances the antigen-presenting function in infected DCs (193), and that local injection of CXCL10 significantly delays the onset of cutaneous lesions (52). By using an *in vitro* infection system, we observed a rapid induction of CXCL10 (4 h for RNA and 24 h for protein) in response to *Lb* infection, even though the levels of IFN-γ were relatively low and somewhat variable (34.83 ± 25.3 pg/ml, Fig. 4.3). Several studies have reported an alternative or IFN-γ-independent mechanism for the induction of CXCL10 production, since CXCL10 can be induced in human monocytes after viral infection by autocrine and/or paracrine action of type I IFNs (190).

The biological effect of CXCL10 is also regulated by the inducible expression of CXCR3 on the surface of target cells (monocytes and T cells) (185). The necessity of CXCR3 for host defense against *L. major* infection in mice is supported by findings that $CXCR3^{-/-}$ mice fail to control *L. major* infection, even in the presence of an efficient Th1 immune response (194, 195). In a self-healing infection model with *L. major*, it has been reported that approximately 20% of lesion-derived cells expressed CXCR3, and that the majority of these CXCR3-expressing cells were neither $CD4^+$ nor $CD8^+$ T cells (194). With regard to human leishmaniasis, expression of CXCR3 on lesion-derived cells has yet to be determined. Our *in vitro* infection data provide solid evidence that $CD14^+$ monocytes are the major target cells for *Leishmania* infection, the predominant producers of CXCL10 (Fig. 4.4), and the principal cell type expressing CXCR3 (Fig. 4.5). Given that over 95% of monocytes contained intracellular ($CFSE^+$) parasites, but only a portion of cells expressed CXCR3 (on average 33% in *La* infection and 47% in *Lb* infection, respectively, Fig. 4.5B), studies are ongoing in the lab to examine the expression of CXCL10/CXCR3 in *Leishmania*-infected tissues.

A functional readout for CXCL10/CXCR3 in host responses is cell migration in transwell system. Different from previously reported studies that have examined cell migration within short periods of time (196), our assay analyzed migrated cells at 14 h of host-parasite interaction in order to provide *Lb*-infected cells the necessary time to produce chemoattractant proteins. Our findings that *Lb*-infected cells can induce the

migration of donor-matched naïve cells through transwell membranes suggest a selective cellular recruitment during active infection. The importance of our findings is that, within the cells that migrated in response to chemotactic gradients, *Lb*-induced attractant factors favored the recruitment of $CD14^+$ monocytes (Fig. 4.6B). Given that blood monocytes are the precursors of tissue MΦs (197) and that MΦs are the ultimate host cell for *Leishmania* survival and replication (198), this selective recruitment of monocytes may lead to an increased availability of new and safe targets for *Leishmania* infection.

The implication of CXCL10-mediated cell recruitment in leishmaniasis warrants further investigation. In an attempt to encourage this direction, we examined the serum levels of this and other proinflammatory mediators in 27 ATL patients. ATL is endemic in most countries of Latin America and can be caused by up to 11 different species of *Leishmania* (15). In Peru, although 3 species of the *Viannia* subgenus [*Lb, L.* (*V.*) *peruviana,* and *L.* (*V.*) *guyanensis*] are of epidemiological importance for ATL, *Lb* is the principal etiological agent for ML (171, 199). Our quantification of inflammatory factors in the sera of ATL patients revealed increased amounts of CXCL10, CCL4 and sTNFRII, especially in the sera of ML patients. These results, together with those obtained from *in vitro* infection in PBMCs (Figs. 4.2 and 4.3) and *in vivo* studies in mice (Fig. 4.1), led us to propose that the elevated production of CXCL10, CCL4 and sTNFRII contributes to extensive cell recruitment/activation and tissue damage. In support of our view, Hailu *et al.* reported that patients with active visceral leishmaniasis had significantly higher serum levels of CXCL10, IL-15, IL-8, IFN-γ and IL-12p40 when compared to those in

asymptomatic *Leishmania*-infected subjects, malaria patients or healthy controls (200). Similarly, a recent study of pulmonary tuberculosis revealed a positive correlation between increased serum levels of chemokines (CXCL9, CXCL10, CCL5 and IL-8) and the active status of the disease, as well as a positive correlation between the high levels of CCR1, CCR2 and CXCR2 on the surface of T and NK cells and disease severity (191). Over-production of CXCL10 has also been associated with other pathological conditions such as chronic obstructive pulmonary disease (201), human rhinovirus-induced respiratory infections (190), and HIV-associated dementia (202).

In summary, this study highlights the potential importance of CXCL10/CXCR3 expression during *Leishmania* infection. For a better understanding of the roles of CXCL10 and CXCR3 during active leishmaniasis, further studies are underway to analyze the expression profiles of these molecules in blood and lesion-derived cells obtained from ATL patients. Collectively, our data suggest that *Lb* infection induces the production of several proinflammatory mediators in human monocytes, and that these factors can efficiently recruit potential target cells for propagating the infection. Moreover, the observation of a similar increase of inflammatory mediators in the serum of active ATL patients, especially among ML patients, suggests a potential role of these molecules in promoting cell recruitment, tissue damage and disease severity.

CHAPTER 5: SUMMARY AND DISCUSSION

Most of the experimental data compiled for this dissertation has been presented as independent manuscripts in Chapters 2, 3 and 4. The results and conclusions of each chapter have been discussed therein. In this final chapter, it is my wish to integrate these findings, present additional unpublished data, and discuss the relevance of my dissertation in a broader context of *Leishmania* infection. Furthermore, I will elaborate on future research directions that could be followed as a result of this dissertation.

DENDRITIC CELL ACTIVATION AND ISG15 FUNCTION DURING *LEISHMANIA* INFECTION

As previously discussed in Chapters 2 and 3, DCs play a pivotal role during the recognition of *Lb* parasites, not only by direct parasite uptake and production of immunomodulatory cytokines (Figs. 2.1 and 3.1), but also by priming of specialized cytokine-producing subsets of T cells (Figs. 2.6 and 3.4). Over the years, several studies have analyzed the interaction between New World species of *Leishmania* and DCs and concluded that modulation of DC activation is parasite species-specific (82). Generally speaking, parasite uptake by immature DCs can lead to 2 possible outcomes: 1) DCs efficiently recognize the parasite and mature into potent Th1-inducing APCs (increased cytokine production, and co-stimulatory molecule expression on the cell surface) (142),

or 2) parasites use immune evasive strategies for their "silent" entry of target cells and the induction of pathogenic Th2-inducing responses (49, 50). Effective clearance of intracellular pathogens such as *Leishmania*, *Mycobacterium* and *Rickettsia* requires the generation of a strong, IFN-γ-inducing Th1 immune response, which promotes pathogen killing by activating infected cells (203, 204). Before the initiation of this thesis research, it was known that *Lb* can cause self-healing disease in all commonly used inbred strains of mice, and that this "general resistance" was correlated with an elevated production of IFN-γ in the draining LN (43, 141). However, the immunological mechanisms leading to this protective immunity were not clear. Our studies in Chapter 2 described how the capture of *Lb* by BMDCs initiates a complex and *Lb*-specific APC maturation process that ultimately leads to Th1-inducing DCs. Furthermore, the cytokine-producing profiles of $CD4^+$ T cells stimulated *in vitro* by *Lb*-infected DCs was similar to the profile observed on the draining LNs of *Lb*-infected mice. Collectively, our studies in Chapter 2 support the view that innate immune responses at the DC level determine parasite-specific T cell responses and disease outcomes (Fig. 5.1).

One of the novel findings of Chapter 2 was the selective upregulation of ISG15 in *Lb*-infected DCs. ISG15 targets a wide variety of intracellular signaling proteins and modifies them through an ubiquitin-like process (119). The consequences of this modification (known as ISGylation) have only been characterized for a few targets, and include the functional inhibition of enzymes and enhancement of transcription suppressor activity (205). Given that *Lb*, but not *La*, infection induced the transcription of ISG15,

and that ISG15 actively interferes with ubiquitination (206), it is possible to hypothesize that ISGylation during *Lb* infection can prevent "key signaling molecules", that promote

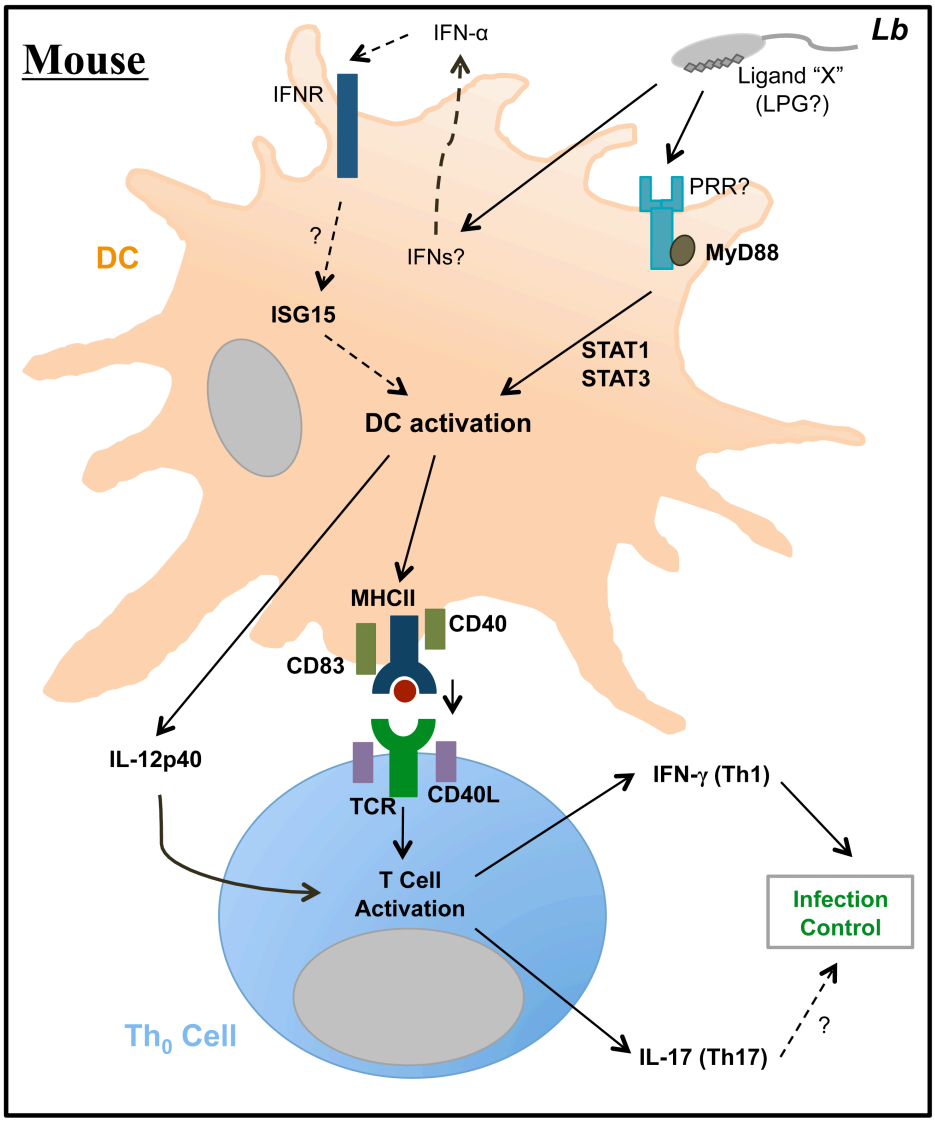

Fig. 5.1 A proposed model for *L. braziliensis* interaction with mouse dendritic cells and its impact on T cell activation. The solid lines indicate events/pathways identified in the present dissertation, or from previously reported in the scientific literature. The dotted lines indicate potential events/pathways that warrant further investigation.

efficient antigen processing, from *Leishmania*-induced proteasomal-degradation (88), and therefore favor parasite Ag presentation and T cell priming. Future studies using ISG15-deficient mice can unravel the signaling requirements for efficient ISG15 upregulation and the biological relevance of this molecule during the immune response against *Leishmania* parasites. Elucidation of the mechanisms leading to ISG15 upregulation in *Lb*-infected cells can potentiate the development of novel drugs to increase ISG15 expression (ISG15 enhancers) and therefore favor the outcome of several viral and parasitic infections. Despite the beneficial roles that ISG15 may have on innate immunity, it is important to mention that because of the inverse correlation between ISGylation and ubiquitination, prolonged expression of ISG15 has been associated with oncogenic malignancies (207). Therefore, the proposed use of ISG15 inducers as therapeutical approaches to combat disease should be carefully addressed.

PATHOGEN RECOGNITION RECEPTORS DURING THE IMMUNE RESPONSE AGAINST *LEISHMANIA* PARASITES

Our results in Chapter 2 revealed that *Lb* infection activates DCs and up-regulates signaling pathways that are essential for the initiation of innate immunity; however, a key question remained: are PRRs involved in *Lb*-mediated DC activation? To address this issue, we generated DCs from MyD88$^{-/-}$ and TLR2$^{-/-}$ mice and examined their responsiveness to parasite infection. Our results suggested that while MyD88 was

necessary for the generation of protective immunity to *Lb*, TLR2 seemed to have a regulatory role during infection. Furthermore, our in detail DC studies showed that immune responses against *La* and *Lb* parasites were differentially regulated, even though these parasites share high genomic similarities and coexist in many of the same geographic areas (208). It could be argued that these two parasite species may have different *in vitro* growth characteristics, and that the immunological behavior observed could be attributed to this difference. Our unpublished data can exclude this argument. As shown in Fig. 5.1A, *La* and *Lb* parasites displayed similar growth rates and also reached the stationary phase at comparable parasite concentrations. It is important to mention that *Lb* parasites have a "longer" stationary phase, which allows them to survive for up to 7 days (in the absence of parasite replication for the last 2-3 days). In contrast, *La* parasites present a relative "short" stationary phase, after which the parasite culture quickly crashes (Fig. 5.1A). To further confirm that the immunological differences between *La* and *Lb* parasites are not generated by the in *vitro* growing conditions, we performed long-term infection cultures in thioglycol-elicited mouse peritoneal MΦs and compared their infectivity over time. As shown in Fig. 5.1B, *La* and *Lb* infectivity rates were comparable both at 24 and 48 h p.i., and slightly decreased for *Lb* parasites at 72 h. Collectively these results indicate that the species-specific immunological events associated with *La* and *Lb* infection of DCs are not due to differences during their *in vitro* growth in the lab.

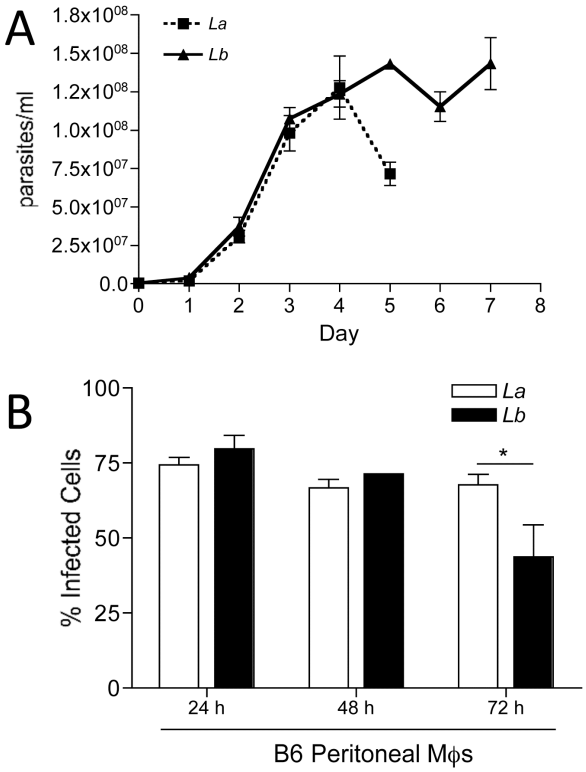

Fig. 5.2 Comparable growth rates and MΦs infectivity between *La* and *Lb* parasites. (A) *La* and *Lb* promastigotes were grown at 23°C in complete Scheider's *Drosophila* medium. Parasite concentration was determined in the culture everyday using a hemocytometer. (B) Peritoneal MΦs were obtained from B6 mice after 5 days of thioglycol elicitation. MΦs were seeded on top of glass cover slips (2×10^5 cells) on 24-well plates and infected with *La* and *Lb* promastigotes at a 10:1 parasite-to-cell ratio at 33°C. At indicated time points, cover slips were recovered, washed and stained with the Diff-Quik staining Kit. The percentage of infected cells was calculated with the following formula: (# infected cells / total # cells x 100). Results pooled from 2 independent experiments are shown. *(p<0.05) indicate statistically significant differences by Student's *t*-Test

Taking into account our studies using DCs from different knockout backgrounds and our observations of comparable parasite growth *in vitro*, we suggest that differential recognition of surface molecules in *La* and *Lb* parasites may potentially explain the immunological differences observed. The surface of *Leishmania* parasites is extensively covered by a coat of GPI-anchored polysaccharide called LPG, which is not only a virulence factor for the parasite, but also has species-specific modifications (138). *Lb* LPG seems to be quite unique, especially because it has been reported to lack side sugar substitutions and to have less LPG in the metacyclic form of the parasite, two characteristics that greatly differ from other *Leishmania* species (140). Despite this, studies analyzing the role of LPG during *Lb* infection and its potential role as a virulence factor have not been performed (Fig. 5.1 Ligand "X"). Because of these apparent gaps in the literature, detailed characterization of the surface components of *Lb* parasites, especially by using LPG-deficient parasites, will be interesting.

Up to now, studies published by our and other labs have identified several TLRs that play an important role during the immune responses against *Leishmania* parasites (134, 178, 209). However, as mentioned in Chapter 1, the PRR family is composed by many other receptors, whose potential role during *Leishmania* has also been extensively studied (210, 211). For example, Chakraborty *et al.* addressed the role of the MR during *Leishmania* infection by using stimuli known to up- or down-regulate the MR expression on MΦs. They reported that dexamethasone-treated MΦs up-regulate expression of the MR and have enhanced uptake of a virulent *L. donovani* strain (212), suggesting a

potential role for this receptor during *Leishmania* infection. Contrarily, Akilov *et al.* showed recently that the clinical course of infection with *L. major* and *L. donovani* was comparable in MR-deficient and WT mice. They concluded that the MR is not essential for the host defense against *Leishmania* parasites (210). Another component of the PRR family that plays an important role in the immune response against *Leishmania* parasites is the complement system. *Leishmania* parasites are capable of avoiding the lytic effects of the complement system, but at the same time, they depend on the opsonic complement coating in order to facilitate their uptake by phagocytic cells (211). Recently, the MΦs receptor with collagenous structure (MARCO) has been characterized on the surface of MΦs. Interestingly, blockage of this receptor using monoclonal antibodies reduced *L. major* promastigotes infectivity by 40%. Similarly, *L. major*-infected CBA/J mice treated with anti-MARCO monoclonal antibodies showed decreased draining lymph node parasite loads and inflammatory responses (213). Thus, it is clear that the role of different PRRs during infection has been elucidated for several *Leishmania* species, but not for *Lb*, and that the complete analysis of PRR involvement during *Lb* infection can shed light into potential therapeutic approaches for the prevention and treatment of ML. More significantly, being *Lb* a self-healing disease in mice, its use in dissecting the innate immune response at the early time points of infection can lead to the discovery of key molecules that might have been overlooked when using more pathogenic parasites.

IL-17 AND ITS IMMUNOTHERAPEUTIC ROLE DURING CUTANEOUS LEISHMANIASIS

Th17 cell differentiation and the potential role for IL-17-producing cells in infectious diseases are active areas of research in recent years (214). Furthermore, investigations looking into the role of IL-17 in leishmaniasis have just started to appear within the last 3 years (51, 142, 215, 216). Importantly, our detailed analysis of DC-*Lb* interactions in Chapters 2 and 3 revealed that IFN-γ, as well as IL-17, play an important role during *Lb* immune responses. As shown in figures 2.6 and 2.7, IL-17 was strongly induced by *Lb* both *in vivo* and *in vitro*, suggesting that Th17 cells may promote the control of *Lb* infection. The suggested role for IL-17 during *Lb* infection became more evident in Chapter 3, where we observed that in the absence of MyD88, the levels of IL-17-producing $CD4^+$ T cells were comparable to those seen in naïve mice, suggesting limited Th17 cell differentiation in infected $MyD88^{-/-}$ mice. We further confirmed these findings by re-stimulating draining LN cells from *Lb*-infected WT and $MyD88^{-/-}$ mice with SLA. As shown in Fig. 5.3A, re-stimulated draining LN cells from *Lb*-infected $MyD88^{-/-}$ mice produced lower levels of IFN-γ and had an almost abolished production of IL-17 when compared to WT counterparts. To further examine the mechanism underlying the abrogated Th17 differentiation, we isolated splenocytes from *Lb*-infected WT and $MyD88^{-/-}$ mice and stimulated them with *Leishmania* antigen under Th17-promoting conditions. Fig. 5.2B shows that even in the presence of IL-6 and TGF-β, splenocytes

from MyD88$^{-/-}$ mice produced significantly lower levels of IL-17 when compared to similarly treated WT splenocytes.

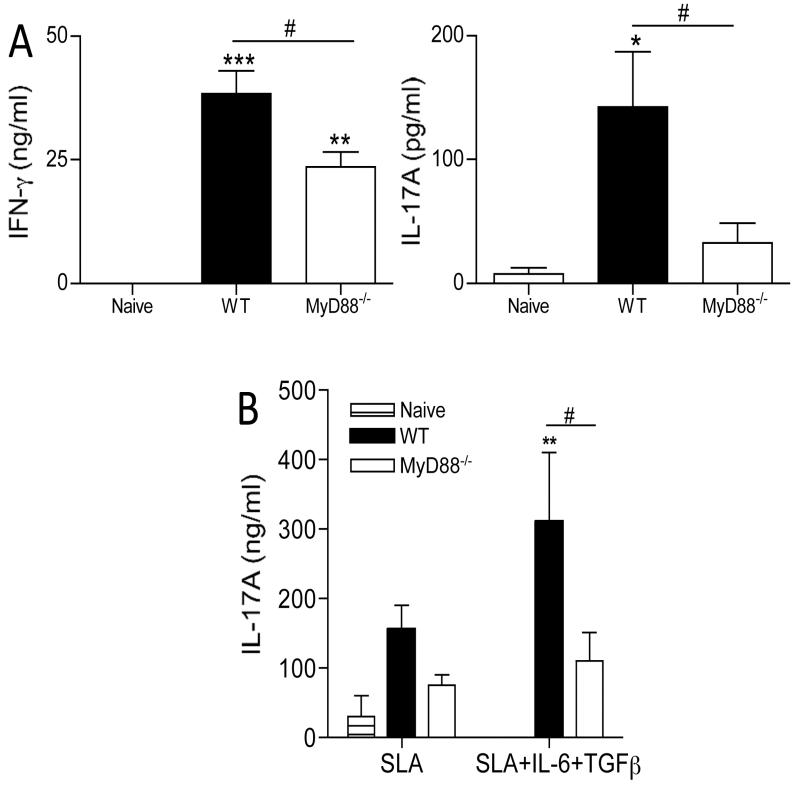

Fig. 5.3 MyD88 deficiency impairs Th17 cells differentiation. MyD88$^{-/-}$ and WT B6 mice (3/group) were infected in the hind foot with 2×10^6 promastigotes of *Lb*. (A) At 8 wk p.i. draining LN cells were collected and restimulated with SLA. (B) At 12 wk p.i., splenocytes were collected and restimulated in the presence of SLA and Th17 differentiating conditions. Levels of indicated cytokines were determined by ELISA. $^{\#}$ ($p<0.05$) represents statistically significant differences between WT and MyD88$^{-/-}$ mice. *($p<0.05$), **($p<0.01$) and ***($p<0.001$) indicate statistically significant differences by Student's *t*-test between the naive and infected groups.

In our hands, IL-17 seems to play a protective role during *Lb* infection; however, recent reports suggest the contrary. Lopez Kostka and colleagues reported that IL-17-deficient BALB/c mice develop smaller cutaneous lesions after infection with highly pathogenic *L. major* parasites, when compared to WT counterparts (215). In this particular model, IL-17 plays a detrimental role by increasing neutrophil migration to the site of infection; an event that is necessary for successful establishment of infection (13). Taking into account our results and those describing the detrimental role of IL-17 during *L. major* infection, it can be hypothesized that IL-17 plays a detrimental role in susceptible models of *Leishmania* infection (such as *L. major*-infected BALB/c mice) (215). However, in self-healing models of disease (such as *Lb*-infected B6 mice), IL-17 could play a protective role by inducing the production of pro-inflammatory cytokines at the site of infection, and therefore favoring parasite clearance (129, 142). Recently, it has been shown PBMCs isolated from individuals that were protected against visceral leishmaniasis produced higher levels of IL-17 and IL-22 when stimulated with *L. donovani* parasites when compared to similarly treated PBMCs obtained from susceptible individuals (216). The above-mentioned study favors our IL-17 hypothesis, suggesting that along with Th1 cytokines, Th17 cells are also associated with protection against *Leishmania* infection. Having shown that Th17 cells are important for anti-*Leishmania* immune responses, it would be of outmost importance to evaluate the susceptibility of IL-17-deficient mice to *Lb* parasites. Furthermore, our results prompt the necessity for animal studies looking at the immunotherapeutic potential of IL-17 during CL, especially in immune deficient models.

HOST AND PARASITE CONTRIBUTIONS TO MUCOCUTANEOUS LEISHMANIASIS

The pathology and clinical course of human leishmaniasis is a complex and multifactorial equation were both host- and parasite-associated factors are involved in the final outcome. Statistically, 80-90% of all human infections with *Leishmania* parasites are asymptomatic. This phenomenon is associated with strong cell-mediated immunity (23) and with the initial encounter between the parasite and the complement system, where the vast majority of the parasites are killed (217). Parasite species plays an important role in disease outcome by predisposing the infected individual to a particular clinical manifestation. However, as explained in Chapter 1, deviations from classical disease are observed routinely (e.g. ML, diffuse CL and post-kala-azar dermal leishmaniasis), suggesting a "genetic predisposition" as a risk factor for severe disease (23). Given that 5-10% of *Lb* infected individuals will develop ML (15), and that ML is characterized by excessive T- and B-cell responses to the parasite, we speculated that the strong but unbalanced production of inflammatory mediators in response to *Lb* infection contributes to cell recruitment and disease severity.

To test this hypothesis, we examined in Chapter 4 the response of healthy volunteer PBMCs to *Lb* infection. Our results suggested that following *Lb* infection, the parasite-induced production of multiple inflammatory mediators by the host might contribute to disease severity by increasing cellular recruitment (Fig. 5.4). Even though

our results suggest that immune responses to *Lb* parasites can explain, at least in part, the high inflammatory characteristics of ML, the contribution of the host genetic factors, particularly during progression from CL to ML need to be addressed.

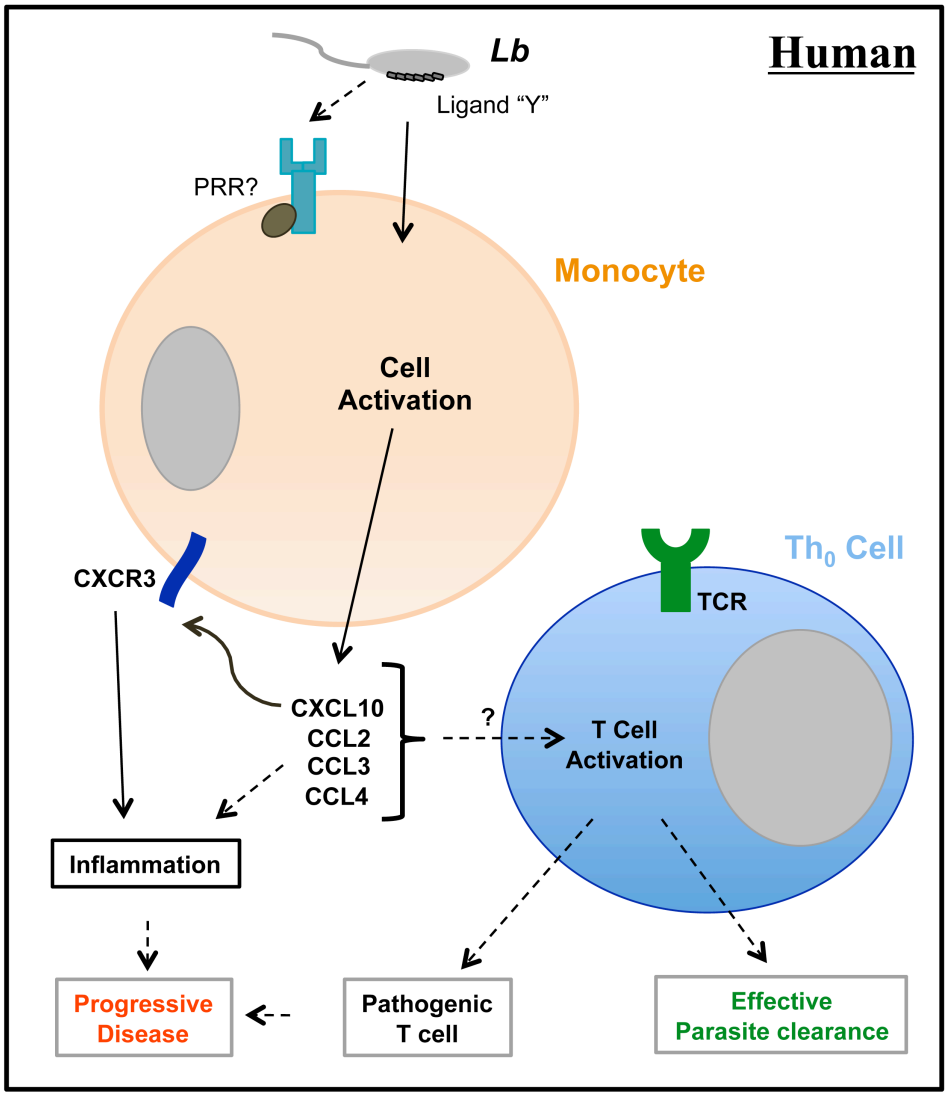

Fig. 5.4 A proposed model for *L. braziliensis* interaction with human monocytes and its potential impact on disease severity. The solid lines indicate events/pathways identified in the present dissertation, or from previously reported in the scientific literature. The dotted lines indicate potential events/pathways that warrant further investigation.

Two models have been proposed in the literature to explain the progression from CL to ML in *Lb*-infected individuals. The first model assumes that all CL patients have latent parasites in the mucosal membranes, and that ML develops from a failure to keep such parasites in check (218). This "failure-in-control-parasite" model is supported by evidence showing that *Leishmania* parasites can be detected in unaffected mucosal tissues of CL patients (219). The second model states that ML arises from the host's failure to control parasite metastasis from the initial cutaneous lesion towards the mucous membrane (220), implying an intrinsic "metastatic" behavior in the parasite. In support of this "metastatic parasite" model, Indiani de Oliveira *et al.* reported that *Lb* clinical isolates from two distinct geographic areas differ in their virulence patterns in BALB/c mice (221), providing strong evidence that high virulent strains of *Lb* could be the cause of ML.

Despite evidence that supports each of these models, it is clear that a third component – the host genetic background – needs to be considered when studying the pathogenesis of ML. The importance of the "host genetic background" is supported by clinical studies showing familial clustering of ML in endemic areas for *Lb* infection in Brazil (222). In recent years, studies have reported significant linkage or associations between single nucleotide polymorphism (SNPs) in human populations and the susceptibility to leishmaniasis (23). For example, Salhi *et al.* reported that the C allele in the IL-10 -819C/T polymorphism increased the risk of lesions in populations exposed to *Lb* infection, and that this risk was directly correlated to an increased IL-10 production

(223). Moreover, SNPs have also been used to predict the "risk" for CL to ML conversion in *Lb*-infected human populations. Castellucci *et al.* associated the IL-6 -174G/C SNP with an enhance risk to develop ML, due in part to a decreased MΦ capacity to produce IL-6 (224). Similarly, when studying the TNF-α 308A/G polymorphism in a Venezuelan population, Cabrera *at. al*. reported a 7.5-fold increase risk of developing ML for *Lb*-infected individuals with the TNF-α 308A/A (TNF2) allele (225).

ML is a disease strongly associated not only with extensive tissue damage, but also with treatment failure (226). Therefore, there is an increasing need for the finding of indicators that might associate phenotypic changes in blood cell composition (e.g., leukocyte surface molecule expression and circulating cytokine profiles) with disease severity and treatment outcome. With the correct scientific and clinical validations, circulating proinflammatory chemokine levels, SNPs and the presence of specific circulating cell phenotypes can all become optimal surrogate markers for the prognosis of ML. As discussed in this section, ML seems to be a complex story where both parasite and host genetics play an important role in the pathogenesis of the disease. Therefore, future research should consider both host- and parasite-associated "genetic traits" as important markers to use and exploit when conducting leishmaniasis research.

CONCLUDING REMARKS

The present dissertation was aimed at improving the understanding of immune processes that take place during *Lb* infection. Our results have revealed three unique features of *Lb* infection. First, using a mouse model we showed how DCs can efficiently recognize, process and present parasite antigens to $CD4^+$ T cells in order to initiate protective immune responses. Second, we demonstrated that interaction between PRRs and *Lb* parasites takes place both *in vitro* and *in vivo*, and that these interactions can modify the outcome of infection. Finally, when evaluating the early events associated with human infection, we revealed a probable association between the magnitude of the inflammatory response generated against the parasite and its potential effect on disease severity. Collectively, these studies have improved our understanding of the pathogenesis of leishmaniasis, in particular of *Lb*-associated CL and ML disease. Our data also reaffirmed the complexity of the disease and ear-tagged several molecules for future studies aiming at better understanding the host- and pathogen-associated mechanisms for pathology and disease control.

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APPENDIX

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Diego Vargas-Inchaustegui
University of Texas Medical Branch
301 University Blvd. Medical Research Bldg. 3.132 Route 1070
Galveston, TX 77555-1070
USA
Phone: 409-772-8163
Fax: 409-747-6869
Email: davargas@utmb.edu

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VITA

Diego Antonio Vargas Inchaustegui was born in Lima, Peru on April 12, 1983 to Percy M. Vargas and Esther R. Inchaustegui. He completed his undergraduate work in Biology at Universidad Peruana Cayetano Heredia (UPCH), in Lima, Peru in 2004. He worked for one year as a Fogarty International Fellow at the Tropical Medicine Institute "Alexander von Humboldt", and in the fall of 2005, he joined the Ph.D. program in the Department of Microbiology and Immunology at UTMB. While at UTMB, he was supported by the James W. McLaughlin Pre-doctoral Fellowship from 2007 to 2009. During his Ph.D. studies, Diego served both as Vice-President (2006) and Treasurer (2007) in the Graduate Student Organization. During his graduate training, he was recognized by different awards such as the Samuel Kaplan Presentation Award (ASM, 2006), the J.W. McLaughlin Travel Award (2007 and 2009) and the Christina Fleischmann Scholarship Award (2008). Upon completion of his Ph.D. studies, Diego will start a Post-doctoral Fellowship at the National Cancer Institute in Bethesda, Maryland. Diego will work under the supervision of Dr. Marjorie Robert-Guroff in the field of HIV vaccine design using replication-competent adenoviral vectors.

Diego A. Vargas Inchaustegui can be contacted through his parents at 101 Casimiro Ulloa, San Miguel, Lima 32, Peru.

This dissertation was typed by Diego A. Vargas Inchaustegui.

EDUCATION

B.S. (Biology), October 2004. UCPH, Lima, Peru.
Lic. (Professional Degree in Biology), July 2008. UPCH, Lima, Peru.

PUBLICATIONS

Vargas-Inchaustegui DA, Hogg AE, Tulliano G, Llanos-Cuentas A, Endsley JJ, Arevalo J and Soong L. CXCL10 production by human monocytes in response to *Leishmania braziliensis* infection. (Manuscript in revision, *Infect. Immun*.)

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ABSTRACTS

Vargas-Inchaustegui DA, Espinosa D, Carlsen E, Tulliano G, Pacheco R, Llanos-Cuentas A, Arevalo J and Soong L. The expression of inflammatory mediators and its association with the susceptibility to mucosal leishmaniasis in Peru. 96th AAI Annual Meeting, Seattle, WA, **2009**.

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ORAL PRESENTATIONS

Vargas-Inchaustegui DA, Xin L, Tai WM, Hogg AE, Corry DB and Soong L. Distinct roles for MyD88 and TLR2 during *L. braziliensis* infection in mice. Annual IHII/McLaughlin Colloquium, UTMB, Galveston, **2009**.

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