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CHARACTERIZATION OF THE EFFECTS OF INTERFERON GAMMA INDUCIBLE PROTEIN-10 (CXCL10) AGAINST THE PROTOZOAN PARASITE *LEISHMANIA AMAZONENSIS*

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by

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Dedication

To my mother whose undying faith in me made this all possible. Thank you for never giving up on me.

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Leishmania amazonensis causes progressive disease in most inbred strains of mice. We have previously shown that *L. amazonensis*-infected C57BL/6 mice have profound impairments in expression of pro-inflammatory cytokines, chemokines and in activation of antigen-specific $CD4^+$ T cells. These impairments are independent of IL-4. The precise mechanism of pathogenesis associated with *L. amazonensis* infection remains largely unresolved. Since chemokines are essential mediators of leukocyte recruitment and effector cell function, we hypothesized that these molecules are important for the initiation of early responses locally and the eventual control of the infection. In this study, we found that CXCL10-treated bone marrow-derived macrophages from both BALB/c and C57BL/6 mice showed decreased numbers of *L. amazonensis* parasites, which was partially due to increased nitric oxide production, as well as elevated production of pro-inflammatory chemokines. When susceptible C57BL/6 mice were locally injected with CXCL10 following *L. amazonensis* infection, there was a significant delay in lesion

development and reduction in parasite burdens, accompanied by a 7- and 3.5-fold increase in IFN-γ and IL-12 secretion, respectively, in re-stimulated lymph node cells. This study confirms that CXCL10 assists in the reduction of intracellular parasites. To address the mechanism underlying this enhanced immunity we utilized stationary promastigotes to infect bone marrow-derived DCs of C57BL/6 mice and assessed the activation of DC subsets and the capacity of these DC subsets in priming $CD4^+$ T cells *in vitro*. We found that CXCL10 induced IL-12p40, but reduced IL-10 production in DCs. Yet, *L. amazonensis*-infected DCs produced elevated levels of IL-10, despite CXCL10 treatment. Elimination of endogenous IL-10 led to increased responsiveness to CXCL10 treatment, as judged by increased IL-12 production in DCs, as well as increased proliferation and IFN-γ production by $CD4^+$ T cells. In addition, CXCL10-treated $CD4^+$ T cells became more responsive to IL-12 via increased expression of the IL-12Rβ_2 chain and produced elevated IFN-γ. This study indicates the interplay between CXCL10 and IL-10 in the generation of Th1-favored, pro-inflammatory responses and further highlights the utility of CXCL10 as a potential therapeutic for the control of non-healing cutaneous leishmaniasis.

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CHAPTER 1: *LEISHMANIA* AND LEISHMANIASIS

HISTORY AND EPIDEMIOLOGY

The beginning of the 20th century was the advent of numerous technological and societal changes whose influence has carried into the present time. Despite the grand expansion of human understanding and means to examine the natural world, the scourge of infectious diseases was and still remains a difficult reality that is combated daily in every corner of the globe. It was in 1901 when British army physician, Sir William Leishman first observed ovoid bodies in the spleen of a British soldier who died from what he described as Dum-dum fever (Leishman, 1903). A few months after reporting Leishman's observations, Charles Donovan published similar results, and the pathogen species was subsequently named *Leishmania donovani*. These 'Leishman-Donovan bodies' would later be renamed as amastigotes, the tissue-infective form of the parasite. Little did either gentleman know that their groundbreaking observations would open the door to over a century's worth of work that employs novel technologies to further describe the nature of a microorganism responsible for disease found throughout the world.

Approximately 12 million people are infected with *Leishmania* worldwide, with nearly 350 million at risk for infection. Leishmaniasis is endemic in more than 88 countries including: Southern Europe, North Africa, the Middle East, Central and South America, and the Indian subcontinent (1). Up to now, the parasite is not endemic in either Southeast Asia or Australia (2). The burden of cutaneous leishmaniasis (90% of cases) is mainly borne by Afghanistan, Pakistan, Syria, Saudi Arabia, Algeria, Iran, Brazil and Peru (3). Over the last decade, migratory trends among populations due to

urbanization or political conflicts, a notable increase in the incidence of cutaneous leishmaniasis has been observed.

The various species of *Leishmania* are segregated by their global location, referred to as Old or New World. Over 20 species and subspecies are capable of infecting humans, which are divided into four distinct categories, according to their key clinical manifestations. They range in severity from self-healing cutaneous ulcers caused by *L. amazonensis, L. brazilensis* and *L. mexicana* in the New World, and *L. major*, *L. tropica*, and *L. aethiopica* in the Old World (2). Mucocutaneous leishmaniasis, which is mainly found in the New World, is caused primarily by *L. braziliensis*. Visceral leishmaniasis results from infection by *L. donovani* and *L. infantum* in the Old World, and *L. chagasi* in the New World (4). Diffuse cutaneous leishmaniasis (DCL) occurs in the New World and is caused by *L. mexicana*, *L. amazonensis*, and on rare occasions, by *L. braziliensis*. Analyses of the phylogenetic relationships between the different parasite species based on numerious taxonomic approaches have illustrated a divergence between the *Leishmania* complexes [i.e. *L. major L. mexicana*, *L. donovani*, and *Leishmania* (Viannia) subgenus] approximately 40–80 million years ago (5), which is in the range of the divergence of mammalian orders (6). Thus, the cutaneous disease-causing species (New World vs. Old World) are as different from one another as they are from those causing visceral disease.

LEISHMANIA LIFE CYCLE

The leishmaniases are insect-born diseases, and their sandfly vectors mainly determine the geographical distribution of *Leishmania* parasites. Presently, about 30 sandfly species have been established as *Leishmania*-transmitting vectors (7). Old and

New World *Leishmania* parasites are carried by the sandfly species *Phlebotomus* and *Lutzomyia,* respectively, and are maintained in reservoir vertebrate hosts such as dogs and woodrats (8). Transmission of *Leishmania* parasites to humans may be anthroponotic (human to human) or zoonotic (animal to human). Elimination of the vector is often the main focus in order to control leishmaniasis outbreaks; however, this intervention is only transitorily effective, not to mention costly, and the employed pesticides often incur harmful effects upon the target environment.

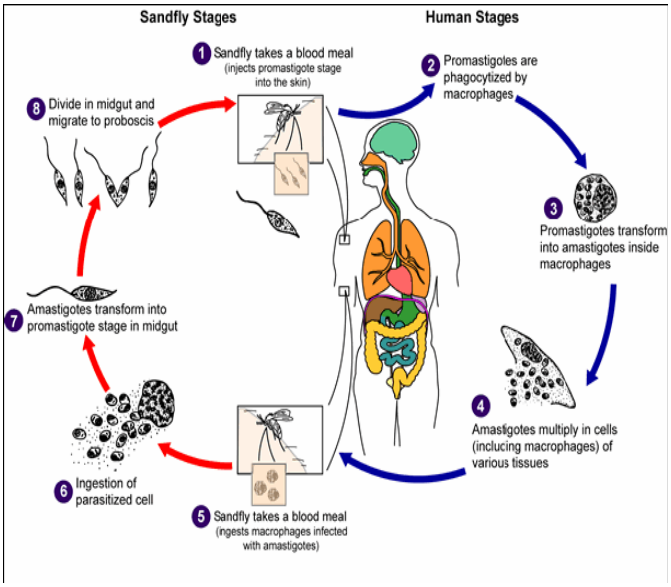

Figure 1.1 *Leishmania* parasite life cycle. *Leishmania* parasites are transmitted by the bite of a female sandfly. During feeding, sandflies inject infective promastigotes (1), which are phagocytosed by MΦs recruited to the site of the wound (2). Flagellated promastigotes eventually transform into a flagellated amastigotes inside phagolysosomes of MΦs (3). Amastigotes multiply in infected cells and affect different tissues, depending mainly on the parasite species (4). Sandflies become infected during blood meals on an infected host when they ingest MΦs that contain amastigotes (5, 6). In the Sandfly's midgut, amastigotes transform back into promastigotes (7), which multiply and then migrate to the stomodeal valve and the proboscis (8). Diagram modified from the Parasite Image Library, WHO/TDR.

When a *Phelbotomus* or *Lutzomya* sandfly ingests amastigotes along with a blood meal, the parasites lodge in the midgut and are rapidly encased by the peritrophic membrane, a chitinous matrix that is secreted by gut epithelial cells. This membrane eventually breaks down, allowing the parasites to progress from the anterior abdominal midgut to the thoracic midgut. Promastigotes then transform from short, ovoid procyclic into slender nectomonads in the thoracic midgut, which quickly blocks the gut of the insect, facilitating inoculation of *Leishmania* during the next blood feeding (9). As migration proceeds to the stomodeal valve and the proboscis, the nectomonads transform

into broad haptomonads, rounded paramastigotes, and slender, infective metacyclic promastigotes (10). The actual number of parasites delivered during the feeding of an infected sandfly is estimated to be roughly 100-1000 (11).

Promastigotes are phagocytosed by cells of the macrophage (MΦ) lineage upon inoculation during a blood meal. Once inside, promastigotes lose their flagella and become amastigotes. *Leishmania* parasites live within the MΦs as round, non-motile amastigotes (3-7 μm in diameter) and are thought to replicate by binary fission within the phagolysosomal compartment. Although there have been some reports of sexual interactions and genetic recombination (12), the evidence for sexual reproduction is relatively weak. Therefore, if such events do occur, they are extremely rare.

MACROPHAGE-*LEISHMANIA* INTERACTIONS

Promastigotes can utilize multiple host cell surface molecules to gain entry. For example, *Leishmania* promastigotes bind to surface molecules such as complement receptor 1 and 3 (CR1&3) and C3b of MΦs before they are internalized. CR1 constitutes the major MΦ ligand for mature promastigotes; however, additional parasite surface glycoprotein (e.g., gp63 membrane protease) and other MΦ receptors (e.g., CR3, mannose fucose receptor) have been identified as important during this process (13-15). Once internalized, promastigotes transform into intracellular amastigotes, eventually rupturing the MΦ and spreading to uninfected cells. The internalization pathway of amastigotes to the MΦs remains largely undefined; however, DC specific intracellular adhesion molecule 3-grabbing nonintegrin (DC-SIGN) has been reported to be the receptor utilized for *Leishmania* amastigote entry (16). The functional consequences in DC after *Leishmania* infections appear to depend on species of *Leishmania*, which can

enhance the surface expression of co-stimulatory molecules and CD40-ligand-induced IL-12 production in DCs (16). Further discussion of the role of DCs in *L. amazonensis* infections will be addressed in the next section.

Leishmania promastigotes are covered with a dense surface glycocalyx, composed largely of molecules attached by glycosylphosphatidylinositol (GPI) anchor (17). These GPI anchored molecules include proteins such as the parasite surface protease gp63 and proteophosphoglycans (PPGs). The most abundant constituent is the large GPI-anchored lipophosphoglycan (LPG) (18). LPG and gp63 are primarily responsible for the virulence of the parasite. LPG has been demonstrated to be crucial for the establishment of infection in the MΦ, as well as for the survival of the parasite while in its sandfly vector (19). Although amastigotes express no or minimal LPG, gp63 facilitates promastigote entry in the host cells, aiding its survival within the mammalian host.

After internalization into phagosomes, secondary lysosomes helps form the complete parasitophorous vacuole (PV). Metacyclic promastigotes rapidly transform into intracellular amastigotes by shedding the promastigote LPG, which migrated to the surface of the infected MΦs. LPG inhibits the respiratory burst, a natural process that occurs after phagocytosis, and the hydrolytic activity of the lysosomal enzymes, possibly through chelation of calcium and inhibition of protein kinase C (20). In contrast to *Trypanasoma cruzi*, the phagolysosome environment is not hostile to *L. amazonensis*, and the amastigotes not only survive, but they can replicate inside these acidic PVs without escaping into the cytoplasm (21). Eventually, the vacuole matures into a late endosome. Association of MHC class II molecules to the PV is critical for $CD4^+$-dependent immune response directed against intracellular *Leishmania* (22, 23). Finally, both the vacuolar and

plasma membranes are disrupted to release the amastigotes, which then are free to infect adjacent cells.

Activation of MΦs is essential for parasite clearance. Indeed, IFN-γ and TNF-α are critical during early infection since both synergize to elicit MΦ NO production which kills intracellular parasites (24, 25). Although both reactive oxygen species and NO were shown to be operating during early *Leishmania* infection, only NO is required for parasite killing (26). In contrast, Th2 cytokines can inhibit NO production in IFN-γ- and TNF-α-activated MΦs, preventing intracellular killing of parasites (27-29). The importance of Th1 and Th2 cytokines in MΦ activation and *Leishmania* infection is apparent. Nevertheless, caution should be maintained when trying to apply generalizations to all *Leishmania* species, specifically *L. amazonensis*. Although *L. amazonensis* promastigotes can be killed by IFN-γ-activated MΦs (30), our lab has recently observed enhanced amastigote replication in IFN-γ-treated MΦs (31), which may be due to suboptimal activation, favoring *L. amazonensis* use of L-arginine as a growth factor (32). Attempts at bolstering MΦ NO production, as well as additional anti-*Leishmania* effector molecules is of particular importance throughout this dissertation study which was experimentally evaluated and will be thoroughly discussed in Chapter 3.

DENDRITIC CELL-*LEISHMANIA* INTERACTIONS

DCs are known as professional APCs and are capable of inducing T cell activation efficiently. Also, DCs are the source of Th cell differentiating cytokines such as IL-12 (33), IL-10 (34), as well as chemokines such as CCL2 and CXCL10 (35). Incubation of *Leishmania* promastigotes with DCs induced early IL-12 production *in vitro*, which is key for the early initiation of T cell immune response in *Leishmania*

infections. In addition, uptake of *Leishmania* amastigotes by skin-derived DCs induced IL-12p70, and lead to the upregulation of co-stimulatory markers against *L. major* infection, contrary to the IL-12 inhibition that occurs in MΦs (36). $CCR2^{null}$ mice were found to be defective in DC migration from the marginal zone of lymph node to the T cell area, resulting in a significant impairment of antigen-specific T cell activation in an otherwise resistant mouse strain to become susceptible to *L. major* (37). Another group described a parasite-mediated down-regulation of CCR7, a crucial DC maturation and homing marker, by *L. donovani*, which allowed the parasite to avoid the host immune response, leading to continued disease syndromes (38). These previous findings imply that the regulatory role of chemokines and their receptors are critical in parasitic infections.

DCs are potent candidates for immunotherapy of leishmaniasis. It has been well-established that DC-derived IL-12 triggers protection against *Leishmania* (39). Indeed, antigen-pulsed Langerhans cells from $IL\text{-}12^{null}$ mice were unable to mediate protection against *L. major* infections (40). DC-based immunotherapy combined with chemotherapeutic agent such as sodium antimony gluconate conferred protection against an established *L. donovan*i infection through involking Th1 immunity (41). Other approaches have utilized a combination of *Leishmania* analogue of the receptors of activated C kinase (LACK) antigen and CpG ODN leading to an increased presence of $CD11c^{+}$ DCs in the draining lymph node (DLN), which vaccinated naive mice in the absence of further antigen and adjuvant, implying an immunotherapeutic potential of $CD11c^{+}$ DCs in *L. major* infection (42).

The role of DCs and their potential as immunotherapeutic agents against *L.*

amazonensis has met with considerable difficulties. Since progressive disease following *L. amazonensis* infection requires primed $CD4^+$ T cells, our lab found that while amastigotes and metacylics efficiently enter and activate DCs, infection with amastigotes failed to induce CD40-depedent IL-12 production, but instead, potentiated IL-4 production (43). Upon transfer into syngeneic recipients, amastigote-exposed BALB/c DCs primed parasite-specific Th cells to produce significantly higher levels of IL-4 and IL-10 than their C3H/HeJ counterparts. Transfer studies with IL-4^{null} DCs indicated that enhanced Th2 priming in BALB/c mice is partially due to IL-4 production triggered by amastigote-carrying DCs, suggesting that *L. amazonensis* amastigotes may condition DCs of a susceptible host to favor activation of pathogenic $CD4^+$ T cells (43).

Recently, our lab observed that *L. amazonensis* parasites have evolved a unique mechanism to induce "semi-activated" DCs, leading to the expansion of a novel phenotype of pathogenic $CD4^+$ T cells (Xin and Soong, in press). Although exogenous IL-1β promoted the activation of parasite-specific $CD4^+$ effector T cells through the enhancement of the antigen-presenting functions of DCs, the intrinsic defects in *L. amazonensis*-infected DCs could not be overcome. Vanloubbeeck et al. (2004) attempted to utilize *L. amazonensis*-antigen pulsed DCs as a potential immunotherapy, and provided data indicating a local impairment of antigen-specific IFN-γ production in the DLN of mice chronically infected with *L. amazonensis*. This defective response continued despite induction of Th1 $CD4^+$ T cells within the DLN, suggesting that a pro-inflammatory, Th1-biased phenotype may not always be indicative of protection to *L. amazonensis*, which has been readily observed in *L. major* (44). Therefore, uncontrolled inflammation, as well as alterations in immune regulation may exacerbate disease syndromes in *L. amazonensis* rather than result in their amelioration, and we will discuss this in further detail in

Chapter 5. Still, more investigation is required to understand the intricate nature of the DC-parasite interaction and how *Leishmania* has learned to cope with the vast repertoire of anti-pathogen responses triggered by the host. Also, attempts to bolster DC Th1-biased functions during *L. amazonensis* will be evaluated and discussed in detail in Chapter 4.

IMMUNOEVASION

Throughout the course of evolution, infectious microorganisms have adopted numerous strategies in order to ensure their survival within host organisms. Indeed, *Leishmania* has efficiently developed the means to circumvent multiple host defense mechanisms. *Leishmania* parasites can survive and successfully establish infection by preventing the activation of the host immune response. In *L. amazonensis* infections, both promastigotes and amastigotes can enter DCs without activating or inducing cell maturation, since key activation markers (i.e. CD40, CD54, CD80, CD86 or OX40L) remained unchanged post-infection (45). In addition, *in vivo* infection with low-dose (1000) *L. major* promastigotes experienced a 4-5 week post-infection delay in presentation of disease symptoms, indicating a silent amplification of the parasites was occurring at the initial stage of infection (46). Furthermore, MΦs infected with *L. amazonensis* amastigotes could not induce the production of either IL-10 or IL-12, further supporting the immunoevasion strategy of 'lack of activation' by the parasite (47).

Leishmania has been reported to inhibit cytokine production. Both promastigotes and amastigotes can inhibit IL-12 production, which is necessary for proper Th1

differentiation and subsequent cytokine response (48, 49). Fcγ ligation, which occurs during promastigote and amastigote phagocytosis, induces IL-10, but suppresses IL-12 production, favoring a Th2 environment (50-52). Additionally, some *Leishmania* species (i.e. *L. major* and *L. donovani*) can induce TGF-β, which inhibit MΦ functions (53, 54). The ability to manipulate cytokine production provides *Leishmania* with a clear advantage over its host, further facilitating pathogenesis.

Leishmania parasites apparently do not alter the phagosome since they rapidly fuse with late endosomes or lysosomes (55). This event facilitates the formation of the (PV) that has a low pH and hydrolytic activity. However, lipophosphoglycan (LPG) on the surface of the promastigote can inhibit phagosome maturation (56, 57). This delay may be necessary for promastigote differentiation into the amastigote form, which can withstand the harsh environment of the PV. Interestingly, *L. mexicana* mutants deficient in LPG or other phoshpoglycan molecules exhibit no difference in survival within MΦs in comparison to wild-type parasites (58), suggesting this species displays redundant systems or uses other unique mechanisms as a means of survival in the PV.

Leishmania may also alter or inhibit host cell signaling pathways as a means of immunoavoidance. In MΦs, inhibition of protein kinase C (PKC) activity and preventing oxidative burst has been reported for *Leishmania* parasites (59, 60). Specifically, *Leishmania* infection of MΦs can significantly suppress the production of NO (61, 62) by the release of anti-inflammatory cytokines (IL-10 and TGF-β) (63), which includes the suppression of the JAK/STAT pathways (62). Interference of JAK/STAT by *Leishmania,*

not only suppresses NO release, but also inhibits IL-12 production (64). *Leishmania*-infected MΦs can also lead to the recruitment of MHC class II molecules to the parasite-containing PVs, where MHC class II molecules are degraded by a parasite-derived cysteine protease (65). Additionally, promastigotes can downregulate myristolated alanine-rich C kinase substrate-related protein (MRP), which may alter vacuolar trafficking or maturation (66-68), favoring parasite replication.

Leishmania parasites can also evade complement-mediated lysis while using complement activation as a mechanism for targeting MΦs for entry. Surface membrane components of metacyclic promastigotes help further prevent insertion of the C5b-C9 membrane attack complex (MAC) (69). The surface proteinase gp63, can cleave C3b to the inactive form, iC3b, thus preventing insertion of MAC. This cleavage also plays a dual role since iC3b can also opsonize parasites for phagocytosis through complement receptors CR1 and CR3 (70).

Leishmania parasites have developed an impressive roster of immunoavoidance mechanisms, allowing them to circumvent and even utilize components of the host immune response in its favor. Further understanding of these processes should be considered when effective therapies against leishmaniasis are designed.

DISEASE MANIFESTATIONS OF *LEISHMANIA*

Depending upon the parasite species, clinical symptoms vary greatly among infected persons, and disease severity can be exacerbated by conditions such as immunosuppresion, malnutrition, or co-infection with other diseases such as HIV (71).

For visceral leishmaniasis in India, Bangladesh, Nepal, Sudan and Brazil, the number of reported cases and geographical areas have increased (3), thus triggering questions concerning the deforestation of previously uninhabited areas, as well as the contribution of global warming to the spread to areas not normally considered endemic for the parasite (72). The incubation period of visceral leishmaniasis varies from 3 to 8 months, (73) and symptoms often include: fever, weight loss, hepatosplenomegaly (usually spleen much larger than the liver), lymphadenopathy, pancytopenia and hypergammaglobulinaemia (74).

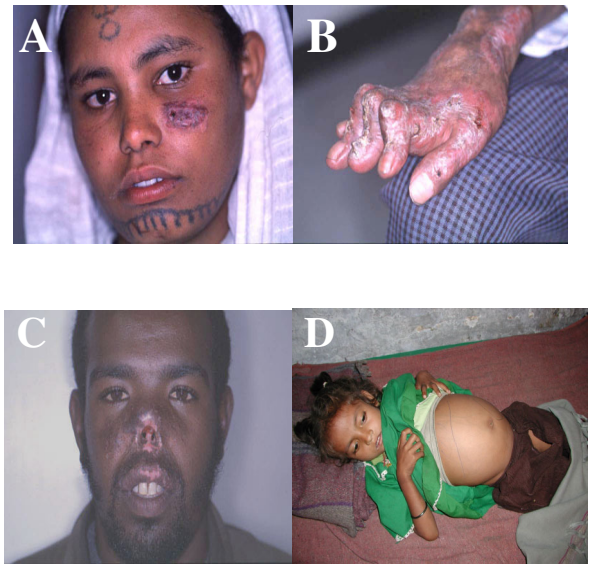

Figure 1.2 Clinical manifestations of leishmaniasis. A. A single ulcerated skin lesion due to cutaneous leishmaniasis. B. Numerous skin lesions due to diffuse cutaneous leishmaniasis. C. Lesions in the facial mucous membranes due to mucocutaneous leishmaniasis. D. Wasting and distended abdomen due to the hepatosplenomegaly seen in visceral leishmaniasis. All photos courtesy of WHO/TDR.

Visceral leishmaniasis can be asymptomatic and self-resolving, but usually becomes chronic and can be fatal without treatment, or despite treatment (75). Death typically occurs because of severe secondary bacterial infections in advanced disease. Some cases of visceral leishmaniasis occur atypically, with reports of the disease also affecting the lungs, pleura, oral mucosa, larynx, esophagus, stomach, small intestine, skin

and bone marrow (76). Treatments are often inadequate, expensive, and too often, toxic to the recipients. Although visceral leishmaniasis is a global concern, with wide ranging social and economic problems, the main focus of this dissertation will be on cutaneous leishmaniasis, especially those caused by *L. amazonensis* infection.

EFFICACY OF CURRENT ANTI-*LEISHMANIA* THERAPIES

The recommended drugs for the treatment of both cutaneous and visceral leishmaniasis, the pentavalent antimonials, were first introduced about 60 years ago. During the 1950s, pentamidine was added, followed by amphotericin B (ampB) in the 1960s (77). For the past 20 years, alternative drugs or new formulations of other standard drugs have become available and registered for use in some countries, with a limited number of novel therapeutics still in clinical trial. The advances in chemotherapy have been significant for visceral leshmaniasis. However, the available drugs are far from ideal; the aim remains to identify cheap and safe drugs that can be utilized for both treatment and disease control. It is unlikely that one single drug will be effective against all forms of leishmaniasis since (a) the visceral and cutaneous sites of infection impose varying pharmacokinetic requirements on the drugs to be used, and (b) there are considerable differences in drug sensitivity among the various *Leishmania* species known to infect humans. In addition, depending on the *Leishmania* species involved, a complicated form of leishmaniasis may develop which requires atypical therapeutic interventions.

Novel treatments face several obstacles, including: (1) the need for drugs where

acquired resistance to the pentavalent antimonials has arisen (77), and (2) the need for treatments for both cutaneous and visceral leishmaniasis in immunosuppressed patients, especially those co-infected with HIV, which results in an exacerbation of disease or emergence from latent infection due to the depleted immune response. In this latter case standard chemotherapy is frequently unsuccessful (71). Although numerous drugs have been used in the treatment of cutaneous leishmaniasis, many have proved to give equivocal results or were effective against some forms of the disease, but not others. For example, the variation in clinical responses to the standard pentavalent antimonials, SSG (Pentostam) and meglumine antimoniate (Glucantime) has been a persistent problem in the treatment of leishmaniasis over the past half-century. The absence of controlled clinical trials to confirm the efficacy of these drugs has been compounded by problems associated with species variation in drug sensitivity (78). Intrinsic variation in the sensitivity of *Leishmania* species to pentavalent antimonials has been shown *in vitro*, where *L. donovani* and *L. brasiliensis* were five-fold more sensitive to drug-mediated parasite killing than *L. major*, *L. tropica* and *L. mexicana* (79). In one of the few controlled clinical trials, the cure rate of antimonials in cutaneous leishmaniasis caused by different species was significantly higher for *L. braziliensis* lesions than for *L. mexicana* lesions (78).

Amphotericin B (AmpB) deoxycholateis considered to be a second-line drug for cutaneous leishmaniasis, and especially for mucocutaneous disease unresponsive to other drugs. Novel ampB formulations have been used successfully to treat cutaneous leishmaniasis, in immunocompromised patients, as well as in pediatric cases (80).

However, since Old World cutaneous leishmaniasis is typically self-limiting, parental administration, cost and toxicity have limited the evaluation of ampB as an additional treatment option.

Miltefosine was first shown to be active against cutaneous leishmaniasis in a Phase II trial in Colombia where *L. panamensis* is common. Cure rates for miltefosine were approximately 91% (40 of 44 patients), whereas in Guatemala, where *L. braziliensis* and *L. mexicana* are common, cure rates using miltefosine achieved only 53% (20 of 38 patients) success and were lower than antimony cure rates of over 90% (81). This pattern of variable responses could be related to drug sensitivity by the individual *Leishmania* parasite species, which has been shown in laboratory studies (82).

Imiquimod is an immunomodulatory imidazoquinoline derivative which is used for the topical treatment of genital warts caused by the human papillomavirus. Imiquimod stimulates a local immune response at the site of application, induces the production of cytokines and NO in MΦs, and has been shown to have an effect in experimental cutaneous leishmaniasis (83). In conjunction with standard antimonial chemotherapy, it has been used to successfully treat patients with cutaneous lesions, who did not respond to antimonial therapy alone (84). Other studies on Old World cutaneous leishmaniasis failed to show that imiquimod was effective as a topical agent alone and further clinical studies are currently ongoing (85). Collectively, these reports suggest that while several treatments are available, none have provided widespread efficacy for use amongst all species of cutaneous disease forms of *Leishmania.* This prompts investigation into novel

approaches for more encompassing treatments.

HUMAN INFECTIONS WITH *LEISHMANIA AMAZONENSIS*

Cutaneous leishmaniasis presents serious medical consequences due to its association with severe scarring and connection with mucocutaneous and variant advanced forms that can be extremely disfiguring. Typically, cutaneous leishmaniasis starts as a papule at the site of a sandfly bite, which then increases in size, crusts and eventually ulcerates. In approximately 90% of cases, these ulcers will heal between 3 to 18 months (76). The incubation period lasts from 2 weeks to several months, and cases up to 3 years have been reported in Old World cutaneous leishmaniasis (73). In contrast, the incubation period of New World cutaneous leishmaniasis is between 2–8 weeks, (86).

Although each species of *Leishmania* are worthy of continued mention, as well as further study, *L. amazonensis, the* etiologic agent for DCL, will be the primary focus of this dissertation. Silva (1945) described the first reported case of DCL in Brazil and for a period of time, *L. amazonensis* infections were considered to be a clinical variety of *L. braziliensis* cases (87). Following studies on the morphology, biochemistry, and behavior of the parasite in laboratory animals and *in vitro* culture, Lainson and Shaw (1972) demonstrated that this parasite was not an isolate of *L. braziliensis,* but rather, a previously uncategorized member of the *L. mexicana* complex. In a subsequent revision of the classification of the neotropical leishmaniasis, the organism was dubbed *L. amazonensis*, within the subgenus *Leishmania* (88). DCL is primarily localized in the Amazon Basin and the Bahia state of Brazil (89-91). In DCL, dissemination of skin lesions rarely occurs over the face and hands and feet, disclosing high parasite numbers owing to poor cell-mediated immune response. Specifically, the delayed-type hypersensitivity (DTH) response to *Leishmania* antigen and the lymphocyte proliferation

assay are always negative in DCL cases, indicating that in these patients the cell-mediated immune mechanisms are incapable of specifically controlling the leishmanial infection (92-94). Also, weak expression of IFN-γ and strong expression of IL-4 (4 times more in some cases) in cutaneous lesions in DCL patients supports the $CD4^+$ Th2 immune response is predominant in DCL disease (95). DCL patients infected with *L. amazonensis* are also likely to have very high antigen-specific $CD4^+$ Th2 immune activation in the lymph nodes, resulting in the proliferation of $CD4^+$ T cells primed to operate as Th2 cytokine-producing cells (mainly IL-4 and IL-10) in the peripheral blood, as well as in the cutaneous lesions. As a result, conventional therapy is very frequently accompanied by relapses of cutaneous lesions of DCL patients (96), due to poor $CD4^+$ Th1 immunity in these cases. DCL is more common in the New World *Leishmania,* but can also occur with *L. aethiopica* in East Africa (97).

Human infection with *L. amazonensis* is less common because the principal sandfly vector, *Lutzomyia flaviscutellata*, is not very anthropophilic and essentially lacks nocturnal activity (98). A substantial increase in the number of *L. amazonensis* infections recorded during the past 15 years might be attributed to improved diagnosis by modern biochemical, serological and molecular biological methods, although there is little difficulty in identifying *L. amazonensis* following its prolific development within the skin of the hamster and in simple blood-agar culture media. A more likely explanation is that drastic ecological changes in the forest environment have increased the type of habitat preferred by the vector and thrown this sandfly into closer contact with humans (99).

Studies involving human infections are often difficult given the geographical remoteness, logistics, and lack of resources in areas where people are at most risk to

infection. This emphasizes the need and importance of mouse models to study, not only the immune responses against *Leishmania* infections, but also design effective therapies that could potentially be utilized in human infection. For the remaining chapters in this dissertation, host responses against *L. amazonensis* will be investigated and discussed in greater detail.

CHAPTER 2: HOST RESPONSES WITH *L. MAJOR* AND *L. AMAZONENSIS*

LEISHMANIA MAJOR ANIMAL MODELS AND TH1/TH2 DICHOTOMY

Development of new treatments to combat leishmaniasis, as well as to further describe the complexities of the drug-immune interface, the animal model is the best choice to approach and answer questions concerning these issues. Cutaneous leishmaniasis caused by *L. major* results in distinctive immunological responses in inbred strains of mice, which determine the outcome of infection. Most strains of mice, such as C57BL/6 mice are typically resistant, whereas BALB/c mice are consistently susceptible (100). The different effector Th1 and Th2 subsets are central in determining pathogenesis and protection in *L. major*-infected mice. These different subsets are generated when naive $CD4^+$ T cells are directed to assume disparate phenotypes by the cytokine milieu present at the time of T cell activation (101).

Protection in *L. major*-infected mice is attributed to the formation of a strong Th1 response. Th1 cells can promote cell-mediated immunity and are capable of secreting MΦ-activating cytokines, including IFN-γ and TNF-α, resulting in the intracellular killing of amastigotes (102). Most critical for Th1 differentiation though is IL-12 secretion; meanwhile, IL-4 is the most influential for Th2 development (103-105). IL-12 strongly induces IFN-γ from NK cells and $CD4^+$ T cells, which can be exogenously administered to drive a Th1 response. Treatment with anti-IL-12 antibody prevents the down-regulation of IL-4, while simultaneously promoting the upregulation of IFN-γ in these mice (106). C57BL/6 mice deficient in IL-12 or its downstream signaling mediator, the signal transducer and activator of transcription 4 (STAT 4), are highly susceptible to *L. major* infection (107). Furthermore, endogenous IL-12 was still required in C57BL/6

mice with healed lesions, since anti-IL-12 treatment reactivated latent parasite growth, which triggered new lesion formation (108). In addition, Th1 cells conferred protection in RAG^{null} (lacking functional B or T cells), C57BL/6 mice against *L. major* infection, only if the recipient mice were not deficient in IL-12 (109). Finally, administration of recombinant IL-12 into susceptible BALB/c mice during the first week of infection diminished Th2 cytokines, while significantly enhanced Th1 responses, leading to the resolution of the infection (110, 111). Collectively, these results demonstrate that IL-12 is the key to inhibit early IL-4 production and is necessary for the induction, as well as the maintenance of a polarized Th1 response following *L. major* infection in C57BL/6 mice.

Progressive skin lesion and subsequent fatal systemic infection in *L. major*-infected BALB/c mice are attributed to the induction of a Th2 response (112), which produces IL-4, IL-5, IL-10 and IL-13, as well as promotes Th2-favoring antibody production. Cytokines associated with a Th2 response favor parasite growth rather than inducing MΦ anti-*Leishmania* effector functions (113, 114). Administration of anti-CD4 antibody (115, 116) and anti-IL-4 antibody (117, 118) was shown to heal *L. major* infection, suggesting that $CD4^+$ population, which induces IL-4 in early infection, plays a critical role in disease progression. Indeed, both IL-4 and IL-10 have been shown to directly enhance replication of *L. major* within MΦs (119). A number of studies also suggest that IL-10 is just as important as IL-4/IL-13 in promoting susceptibility to *L. major* (113, 120). For example, IL-10 has been shown to play a suppressive and/or regulatory role in numerous parasitic diseases, including *Leishmania* infections (63). Administration of anti-IL-10 antibody during *L. major* infection further improved susceptibility to infection in BALB/c mice (113). Also, IL-10 was required for increased

parasite persistence in both resistant C57BL/6 and susceptible BALB/c mice (121). Co-administration of IL-10 plasmid with low dose of *L. major* inoculums that are known to induce protective Th1 phenotype, instead promoted disease in BALB/c mice, further confirming the role of IL-10 in disease progression (122). The role of IL-10 in cutaneous leishmaniasis caused by *L. amazonensis* infection is less clear, and this issue will be addressed in greater detail in Chapter 4.

Differentiation of Th subsets, specifically IL-12 and IL-4 as the driving forces behind development, is crucial in extrapolating data obtained in *Leishmania* experiments in general. Although both reactive oxygen species and NO were shown to be operating during early *Leishmania* infection, NO is considered requisite for parasite killing (26). Since MΦ activation is crucial for parasite clearance, IFN-γ and TNF-α are critical cytokines that need to be present during early infection. Understanding the mechanisms *Leishmania* has evolved to manipulate the host immune response and how these strategies can be exploited is of key interest to this dissertation project.

MOUSE MODELS OF CUTANEOUS LEISHMANIASIS CAUSED BY *L. AMAZONENSIS*

Unlike *L. major* murine infections, most inbred strains of mice are susceptible to *L. amazonensis* infection. Among the different inbred mouse strains, BALB/c mice are the most susceptible to *L. amazonensis*, but all strains of mice that have been studied to date are also susceptible to infection. In contrast to the *L. major* models, *L. amazonensis*-infected BALB/c and C57BL/6 mice do not mount a vigorous Th2 response even though both mouse strains develop progressive skin lesions. Th2-dependent mechanisms cannot completely account for the disease pathogenesis observed during infection by *L. amazonensis*. For example, high levels of IL-4 are not detected in mice during *L.*

amazonensis infection (123), and neutralizing anti-IL-4 treatment is not curative (124). Targeted deletion of the IL-4 or the IL-10 genes in C57BL/6 and C3H mice had minimal effects on lesion development and parasite burdens (125-127). Interestingly, a slight increase in resistance to *L. amazonensis* was enhanced in IL-4Ra^{null} compared with IL-4^{null} mice, indicating that a disease-promoting role for IL-13 during infection does partially contribute to disease progression (128).

L. amazonensis-infected BALB/c and C57BL/6 mice were shown to generate low levels of Th1 cytokines (30, 124, 129); however, this weak Th1 response required for the formation of disease syndromes attributed to *L. amazonensis* infection (30). Lesion development and tissue parasite load are significantly lowered in MHC class II^{null}, RAG2^{null}, and BALB/c severe combined immunodeficiency (SCID) mice, but not MHC Class I^{null} mice (30), suggesting an important contribution to pathogenesis by CD4^{+} T cells. Additionally, infection of IL-10^{null} C57BL/6 mice with *L. amazonensis* experienced a transient enhancement of a Th1 response, at an early state of infection, but this enhanced subsequently diminished during the chronic stage of infection (126). Exogenous administration of IL-12, in both chronically infected C3H and C57BL/6 mice, did not alter the course of infection, implying that *L. amazonensis* can trigger a potent immunomodulatory response, enabling its evasion of widespread killing by the host immune response (126). The lack of an effective Th1 response is not the sole cause for disease susceptibility of mice to *L. amazonensis* infection. and multiple immune factors are highly likely to play an important role.

CD4^{+} T cells have been demonstrated to be more important than CD8^{+} T cells in

the pathogenesis of primary *L. amazonensis* infection (30). However, there is increasing evidence that $CD8^+$ T cells produce IFN-γ during infection and thus may be involved in the protection of immunized animals (130, 131). Adoptive transfer of *in vitro* generated Th1 cells that produced high levels of IFN-γ and TNF-α prior to infection triggered protection in otherwise susceptible mice (127), suggesting a strong, polarized pro-inflammatory response is requisite to resolve *L. amazonensis* infection. Additionally, B cells have also been found to be contributing factors in disease formation. Miles et al. (2005) found that IgG not only failed to provide protection against *L. major*, but also contributed to disease progression (132), similar to *L. amazonensis* infections, in which circulating antibody is required for maintenance of the parasite (133). In general, antibodies may facilitate parasite uptake by MΦs, further enabling the parasite to escape immune-mediated elimination (134).

NOMENCLATURE AND GENERAL FUNCTIONS OF CHEMOKINES

The major role of chemokines is to guide the migration of cells. Chemokines are a superfamily of low molecular weight (6–17 kDa) cytokines that recruit distinct subsets of leukocytes, which are activated through increased adhesion, degranulation and respiratory burst (135). To date, more than 44 different chemokines have been described and there are 21 known chemokine receptors. Most chemokines are secreted proteins of about 67–127 amino acids in size. Their production is stimulated via several sources including, but not limited to: lipopolysaccharides, mitogens, pro-inflammatory cytokines, as well as several pathogens (136). The two major structural subfamilies are differentiated by the arrangement of the two amino-terminal cysteine residues, which are either separated by a single amino acid (CXC) or are in adjacent (CC) positions. C chemokines (which lack two out or four cysteines) and CX3C chemokines (with three

intervening amino acids between the first two cysteines) are minor structural subfamilies (137).

Chemokine actions are mediated via specific cell-surface receptors, which are members of the seven-transmembrane domain G-protein coupled receptor family. The chemokine receptors are named CXC, CC, XC and CX3C, followed by R and a number (according to their ligands: CCR1–10, CXCR1–6, XCR1–2 and CX3CR1). The chemokine–receptor interaction can be described as being considerably promiscuous, in that each receptor interacts with several chemokines, and each chemokine binds to several receptors (138). When chemokines engage their G-protein coupled receptors (GPCR), the heterotrimeric proteins are generally members of the Gαi subfamily of G proteins and are pertussis toxin sensitive (139, 140). The expression pattern of chemokine receptors is heterogeneous among leukocytes, and engagement with their respective ligands regulates cytoskeletal rearrangement, integrin-dependent adhesions, as well as binding and detachment of cells from their substrate. This occurs in a coordinated manner, with extension and retraction of pseudopods to execute coordinated directional migration (141). Currently, the precise mechanism through which cells respond to a chemotactic gradient has yet to be determined.

Most chemokines share the ability to activate G-protein sensitive phospholipase C (PLC) isoforms, resulting in the cleavage of Phosphatidylinositol (4,5)-bisphosphate (PIP2) into two second messenger molecules known as Inositol triphosphate (IP3) and diacylglycerol (DAG) that trigger intracellular signaling events (142). DAG then activates protein kinase C (PKC), and IP3 triggers the release of calcium from intracellular stores (143). These events promote many signaling cascades (such as the

MAP kinase pathway) that generate chemokine-mediated responses such as chemotaxis, degranulation, release of superoxide anions and changes in the avidity of the cell adhesive integrins within the cell harboring the chemokine receptor (144).

The actions of chemokines are specific to particular cellular groups: members of the CXC class act mainly, but are not limited to polymorphonuclear cells (PMNs), whereas members of the CC class act on a larger group of cells, including monocytes, basophils, eosinophils, and lymphocytes, but not PMNs. Lymphotactin, the only C chemokine, acts solely on specific subgroups of B and T cells. Fractalkine, a CX3C-type cytokine, has been reported to attract monocytes, PMNs and T cells (137). Previously, chemokines were grouped into the subfamilies termed 'inflammatory' and 'homeostatic' chemokines. However, several chemokines have been recently shown to possess 'dual-functions.' Inflammatory chemokines have broad target-cell selectivity and act on cells of both the innate and the adaptive immune system. Homeostatic chemokines navigate leukocytes during hematopoiesis. Dual-function chemokines can target non-effector leukocytes and most importantly, participate in immune defense functions (136).

CHEMOKINES AND TH1/TH2 IMMUNITY

Extensive attention has been focused upon Th cell cytokines during polarization and their role in the resolution of *Leishmania* infections. Recent studies have demonstrated that the response of Th1 and Th2 cells to chemokines is also distinct and is primarily based upon the expression of particular chemokine receptors. For example, Th1 cells express CXCR3 and CCR5 (145) and are thereby attracted by CXCL9, CXCL10, and CXCL11, all of which bind to CXCR3 and are induced by IFN-γ. The ligands for

CCR5 are CCL2, CCL4 and CCL5 which are preferentially, but not exclusively, associated with a Th1 response (146). Recently, regulatory DCs were found to secrete a higher level of CXCL10 than immature DCs, and more CXCL10 is produced after stimulation with TLR-2, -4, -3, and -9 ligands (147). Blockade of IFN-α/β inhibited CXCL10 production by TLR agonist-activated regulatory DCs (147), further substantiating the importance of pro-inflammatory chemokines in the process of Th1 development.

Th2 lymphocytes express CCR4 (148), which interact with CCL17 and CCL22. Both CCL17 and CCL22 are released from monocytes that are activated by IL-4 and IL-13 and selectively recruit Th2, rather than Th1 populations of lymphocytes (149). Additionally, $CCL2^{null}$ mice failed to express a Th2 phenotype with low levels of IL-4, IL-5 and IL-10 being produced (150). The control of differentiating uncommitted T cells to the Th1 phenotype is believed determined by the following: interaction with differing subpopulations of DCs; exposure to a milieu of cytokines that suppress one phenotype; or, differences that depend on the nature of the antigenic stimulus (146). In terms of *Leishmania* infections, as mentioned previously in Chapter 2, development of a strong Th1 response leads to a healing phenotype in *L. major*, and potentially in *L. amazonensis* infections, which underscores the importance of pro-inflammatory chemokines in shaping the host response against *Leishmania* infections.

CHEMOKINES AND *LEISHMANIA*

Cytokines are crucial for chemokine production and can precede the expression of some chemokines, inducing the production of additional inflammatory mediators. Cytokines exert a secondary effect on leukocyte recruitment by eliciting the expression of several chemokine genes (151). TNF-α and IL-1β released from activated neutrophils and MΦs contribute to chemokine synthesis in several cell types: neutrophils, fibroblasts, and endothelial and epithelial cells (136). TNF-α and IL-1β, together with CCL3, can regulate Langerhans cell-mediated transport of *Leishmania* from the infected skin to the DLNs in murine cutaneous leishmaniasis caused by *L. major*. (152). IL-12 is required for the induction of Th1-related chemokines such as XCL1, CXCL10, and CCL2 in DLNs of *L. major*-infected, resistant mice (153).

Th1- and Th2-derived cytokines can also have antagonistic effects on chemokines. For example, CXCL9 and CXCL10 are more selectively induced by IFN-γ (154); meanwhile, Th2-related IL-4 and IL-13 preferentially induce CCL22 and CCL6 production in MΦs, which is inhibited by IFN-γ (148, 155). During murine *L. donovani* infections, CCL3, as well as its receptors, CCR2 and CCR5, were found to influence the production of IFN-γ by T cells, suggesting a cross-talk between the TCR and the chemokine receptor signaling pathways (37, 156). Additionally, in *L. donovani*-infected livers, the amount of IFN-γ-producing cells increased when the negative regulator CTLA-4 was blocked, which correlated with enhanced CXCL10 expression (157). At the MΦ level, both CCL2 and CCL3 were able to trigger NO production in *L. donovani*-infected MΦs (158). In *L. mexicana* infection, self-healing, localized lesions showed a high

expression of CCL2, CXCL9, CXCL10 and only low amounts of CCL3 (159). In contrast, lesions from chronic DCL primarily expressed CCL3, suggesting CCL2 and CXCL10 contribute to the healing process by inducing leishmanicidal activities in human monocytes and T cells (160). Although CXCL10 contributes to NK chemotaxis and IFN-γ production in *L. major* infections, it remains unclear if this molecule aids in the development of resistance to other *Leishmania* species. The role of CCL2 is less clear since it has been linked to inhibit IL-12 production, yet it can stimulate monocytic cells to eliminate *L. major* (160, 161). Additionally, $CCR2^{null}$ mice, which experience defective Langerhans cell homing to the DLNs are highly susceptible to *L. major* (162), suggesting, CCL2 plays a prominent role in both cell migration and activation in preventing pathogenesis of cutaneous leishmaniasis (153).

Our lab recently found that *L. amazonensis*-infected C57BL/6 mice had significantly delayed and depressed expression of pro-inflammatory cytokines and chemokines in both foot tissues and DLNs compared to *L. major* infected mice of the same background (127). This suggested that an impairment exists in multiple immune functions at early stages of infection with *L. amazonensis* parasites, which further supports the notion to explore immune augmentation as an intervention against *L. amazonensis* infections. Since IFN-γ is critical for resolution of *Leishmania* infections, factors that enhance this molecule, namely CXCL10, need to be further examined in *L. amazonensis* infections. Defective production of CXCL10 and other chemokines in the DLNs, in conjunction with insufficient NK cell recruitment and/or cytokine production may contribute to the susceptibility seen in other murine models of *Leishmania* (163). The importance of understanding the factors involved in maintenance of chronic disease

is crucial since chronic *Leishmania* infections in humans has been linked with a poor T cell mediated or mixed T cell response (126).

OBJECTIVE OF THIS DISSERTATION

From the preceding discussion, it is apparent that *L. amazonensis* infection can cause progressive disease via multiple mechanisms in most inbred strains of mice. We have previously shown that *L. amazonensis*-infected C57BL/6 mice have profound impairments in the expression of pro-inflammatory cytokines and chemokines, as well as the activation of antigen-specific $CD4^+$ T cells (127, 129). These impairments are independent of IL-4, but are partially due to IL-10 production. Since chemokines mediate leukocyte recruitment and effector cell function, in Chapter 3 we examine the effects CXCL10 on MΦ activation, as well as its ability to combat *L. amazonensis* infection in susceptible mice. We found that *in vitro* treatment of MΦs with CXCL10 significantly reduced parasite burdens in a dose-dependent manner partially via triggering increased production of NO. When susceptible C57BL/6 mice were locally injected with CXCL10 at early stages of *L. amazonensis* infection, there was a significant delay in lesion development and reduction in parasite burdens, accompanied by marked increases in IFN-γ and IL-12 secretion in DLN cells. To further understand the molecular basis of this enhanced resistance in CXCL10-treated mice, in Chapter 4, we examine the effects of CXCL10 treatment on the functions of DC and $CD4^+$ T cells, as well as the interplay between IL-10 and CXCL10 in immune regulation during *L. amazonensis* infection. We found that CXCL10 can induce IL-12p40, but reduces IL-10 production in DCs. Yet, *L. amazonensis*-infected DCs exhibited elevated IL-10, despite CXCL10 treatment. However, *L. amazonensis*-infected, CXCL10-treated $IL\text{-}10^{null}$ DCs produced enhanced IL-12p40, suggesting that the parasite utilizes IL-10 for its advantage within the host.

Additionally, CXCL10-treated $CD4^+$ T cells became more responsive to IL-12 via increased expression of the IL-12Rβ_2 chain, thereby producing elevated IFN-γ. Therefore, exogenous CXCL10 triggers a Th1-favored, pro-inflammatory phenotype, which aids in the reduction of intracellular parasites. This dissertation research highlights the value and molecular basis of CXCL10 treatment, as well as opens new avenues for therapeutic control of non-healing cutaneous leishmaniasis in the New World.

CHAPTER 3: CXCL10/IP-10-MEDIATED PROTECTION AGAINST *L. AMAZONENSIS* INFECTION IN MICE[1]

INTRODUCTION

Leishmania parasites preferentially infect cells of the MΦ lineage and replicate within phagolysosomes, resulting in diverse clinical manifestations. The severity of disease is dependent on both the causative species of parasite, as well as the immunological status of the host (128). Immune regulation of host responses to *Leishmania* has been extensively investigated in murine models of leishmaniasis. Resistance to *L. major* in C57BL/6 (B6) or C3H mice (164) is clearly linked to a dominant Th1 response. While susceptibility of BALB/c mice to *L. major* is reliant upon early production of IL-4, which promotes lesion development during the early stages of infection, long-term persistence of *L. major* in the resistant strains of mice appears to be mediated by IL-10 production (121).

Although the New World species *L. amazonensis* can cause progressive disease in a majority of inbred mouse strains, including BALB/c, B6 and C3H mice (30, 43, 127), these mice do not exhibit a classical Th2-dominant phenotype. Lesion development in *L. amazonensis*-infected mice is largely due to an impairment of parasite-specific Th1 cell activation (30, 43, 127). However, circulating antibodies may also contribute to *L. amazonensis* pathogenesis (133) via multiple mechanisms, including antibody-mediated IL-10 production (68). We and others have previously shown that RAG2^{null} and MHC

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class II^{null} mice are refractory to *L. amazonensis* infection, suggesting an essential role for $CD4^+$ T cell-mediated responses in immunopathogenesis in *L. amazonensis* infection (30, 165).

Attempts at using pro-inflammatory cytokines to boost protective immunity against *L. amazonensis* have yielded limited success. For example, recombinant IL-12 (166) or IL-1α (167), when administered prior to *L. major* infection, made otherwise susceptible BALB/c mice resistant; however, exogenous IL-12 (125) or IL-1α (Li L, Vasquez, RE and Soong L, unpublished results) did not ameliorate *L. amazonensis* disease progression. These negative results are due, in part, to an IL-4-independent down-regulation of the IL-12 receptor β2 chain on $CD4^+$ T cells, making these cells unresponsive to both intrinsic and exogenous cytokines (125). The precise mechanism leading to this IL-12 unresponsiveness remains unresolved.

Chemokines belong to a large subset of cytokines that are crucial mediators for leukocyte function, activation and trafficking of cells involved in inflammatory responses (163). CXCL10/IP-10 (IFN-γ-inducible protein 10) binds with high affinity to CXCR3 (168), a receptor known to be expressed on several types of cells in the hematopoietic lineage, including activated and memory $CD4^+$ and $CD8^+$ T cells, NK cells, and some subsets of dendritic cells (160, 169, 170). Injection of CXCL10 into *L. major*-infected BALB/c mice has been shown to induce strong NK cell recruitment and activation (171). Interestingly, *L. donovani* promastigotes produce a granulocytic chemotactic factor that

preferentially inhibits the production of CXCL10 from polymorphonuclear cells, suggesting a complex regulation of this chemokine in host-parasite interactions (172).

We have recently shown an insufficient induction of pro-inflammatory mediators at early stages of infection with *L. amazonensis* parasites and deficient priming in parasite-specific Th1 cells during the course of infection (127). We hypothesized that administration of CXCL10 would skew the host's local environment towards a Th1-type immune response and therefore facilitate parasite elimination. In the present study, we have shown that treatment with recombinant CXCL10-activated, murine bone marrow-derived macrophages (BM-MΦs) to significantly reduce parasite infection *in vitro*, and that local injection of CXCL10 significantly delayed disease development in susceptible mice. This enhanced parasite killing was partially due to the production of multiple effector molecules such as IFN-γ, IL-12 and NO. Collectively, this study indicates that exogenous CXCL10 enhances protective defense mechanisms that are critical for the control of non-healing, New World cutaneous leishmaniasis.

MATERIALS AND METHODS

Mice: Wild-type BALB/c, C57BL/6 (B6), C3H/HeJ (C3H), as well as $iNOS^{null}$ B6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME). They were maintained under specific pathogen-free conditions and used at 6-10 weeks of age. All protocols were approved by the Animal Care and Use Committee of the University of Texas Medical Branch (Galveston, TX).

Parasites: *L. amazonensis* (MHOM/BR/77/LTB0016) parasites were maintained by regular passage through BALB/c mice. To culture parasites, 20% FBS-supplemented (Hyclone, UT) Schneider's Drosophila medium (Invitrogen, Rockville, MD) was used at pH7 for promastigotes and pH5 for amastigotes. Promastigotes were cultured at 23ºC. Tissue-derived amastigotes were harvested from foot tissues of infected BALB/c mice and cultured at 33ºC for 48 h before *in vitro* infection.

Macrophage culture: BM-MΦs were generated, as previously described (31). Briefly, bone marrow cells were seeded in a Petri dish at 2 x 10^6 per 10 ml of complete IMDM (Iscove's modified DMEM containing 10% FBS, 1 mM sodium pyruvate, 50 μM 2-ME, 50 μg/ml gentamycin and 100 U/ml penicillin) medium supplemented with 10% L929 culture supernatants. After 5 days, non-adherent cells were discarded, and adherent cells were maintained for an additional 4 days before being detached from the Petri dish with cold PBS containing 2 mM EDTA. These cells were washed twice in warm IMDM medium, and were then seeded into 24-well plates at 3 x 10^5 cells/well.

Flow cytometric analysis of macrophage surface antigens: The quality and quantity of BM-MΦs were assessed by FACS, using specific or isotype control mAbs purchased from BD Biosciences (San Jose, CA) unless indicated otherwise. For blocking nonspecific Ab binding and Fc receptors, purified anti-mouse CD16/32 (eBioscience, San Diego, CA), hamster IgG (Pierce, Rockford, IL) and 2% rat serum (Sigma, St. Louis, MO) were used. After 30 min of blocking on ice, cells were stained with PE-conjugated anti-mouse CXCR3 mAb (R&D Systems, Minneapolis, MN) and FITC-conjugated, anti-

mouse F4/80 mAb in a final volume of 200 µl for 30 min (1 µg/10^6 cells). To assess the general quality of BM-MΦ cultures, cells were also stained with FITC-conjugated anti-mouse CD3 and PE-conjugated, anti-mouse CD8α (eBioscience). Cells were washed and analyzed on a FACScan (BD Biosciences, Franklin Lakes, NJ). For characterization of BM-MΦs, at least 10,000 events were collected. Data were analyzed with FlowJo software (TreeStar, San Carlos, CA). Isotype control Abs included: FITC-conjugated hamster IgG1, IgG2a, and PE-conjugated rat IgG2a (eBioscience). BM-MΦ purity was determined to be ~90%, as judged by staining with the F4/80 MΦ specific marker. $CD3^+$ and $CD8\alpha^+$ cells were undetectable via FACS analysis.

Macrophage stimulation and parasite infection: BM-MΦs were cultured for at least 12 h in 24-well plates, washed once with warm medium, and then treated with recombinant CXCL10, CCL2, TNF-α, IFN-γ (Leinco Technologies, St. Louis MO) or LPS (Sigma, St. Louis, MO) at indicated concentrations (see Fig 3.1) and/or in combinations for 4 h before infection with *L. amazonensis* promastigotes or lesion-derived amastigotes. According to the manufacturer's data sheet, endotoxin levels in these recombinants were measured to be less than 0.1 ng per µg. Amastigote binding to MΦs was synchronized by centrifugation of the culture plates at 100 x g for 5 min immediately after the addition of parasites. Parasite-exposed BM-MΦs were kept for 24 h at 33ºC, a temperature consistent with that of *Leishmania*-induced cutaneous lesions, and then moved to 37ºC for the rest of the observation period. Infected BM-MΦ cultures were processed to evaluate intracellular parasite burdens at 5, 24, or 48 h post-infection, as in our previous report (31). Briefly, MΦs in 24-well plates (3 wells per condition) were

gently washed twice with PBS and then exposed to 0.2 ml of 0.01% SDS in PBS at 37ºC. The process of cell lysis, monitored under an inverted microscope, was typically completed within 10 min. The cell suspension was immediately supplemented with 0.8 ml complete culture media to avoid lysis of released parasites. The number of parasites per well was counted with a hemocytometer. In some cases, the numbers of intracellular parasites per cell were assessed by fluorescent microscopy, as in our previously described studies (63). Briefly, BM-MΦs were seeded in 24-well plates (3×10^5 cells/coverslip/well). Cells were untreated or treated with either CXCL10 (100 ng/ml), CCL2 (100 ng/ml), or LPS (20 ng/ml) plus IFN-γ (20 ng/ml or 2 U/ml) for 4 h prior to infection with 2.4×10^6 stationary-phase promastigotes (at 8:1 parasite-to-cell ratio). At 5 or 48 h, cells were fixed on a glass coverslip with methanol at 4°C for 20 min and then washed thrice with PBS. Antisera from *L. amazonensis*-infected BALB/c mice were diluted 1:500 in PBS and used to stain cells for 20 min at 4°C. After washing, cells were stained with FITC-conjugated goat anti-mouse IgG (1:250) at 4°C for 20 min. Cells were counterstained with a mounting medium containing 4',6'-2-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA) and viewed under an Olympus BX51/52 fluorescent microscope (Olympus America Inc., Melville, NY). To enumerate intracellular parasites, the software-merged images from 10 random visual fields per condition were taken for counting total numbers of parasites and host cells. Data are presented as number of parasites per 100 MΦs, and were pooled from three independent experiments (representing a total of 30 visual fields).

Mouse infection and disease evaluation: Mice (5 per group) were subcutaneously (s.c.) inoculated in the right hind foot with 2 x 10^5 *L. amazonensis* metacyclic promastigotes that were purified through negative selection with the 3A1 mAb (a generous gift by Dr. David Sacks, NIAID), as previously described (173). Lesion sizes were measured weekly with a digital caliper (Sigma, St. Louis, MO). At indicated time points, mice were sacrificed to determine the parasite burden by a limiting dilution assay, as previously described (30). For *in vivo* chemokine treatment, each mouse was injected s.c. with CXCL10 (100 ng in 5 μl) or PBS (5 μl) at 1, 3 and 7 days post-infection.

RNA extraction and RT-PCR analysis: Total RNA was isolated from control or CXCL10-treated BM-MΦs or whole foot tissue from sacrificed mice using Tri reagent (Sigma). Splenocytes from naïve or *L. amazonensis*-infected mice were treated with LPS/IFN-γ and served as positive controls. Total RNA (100 ng) was subjected to RT-PCR analysis, with annealing temperatures of 56°C for CXCR3 and 58°C for IFN-γ and β-actin. Primer sequences (listed as 5'-3') were as follows: CXCR3 F: GCTAGATGCCTCGGACTTTG, R: GCTGATCGTAGTTGGCTGATA (175); IFN-γ F: CATTGAAAGCCTAG AAAGT CTG, R: CTCATGGAATGCATCCTTTTTCG (174); β-actin F: CCAGCCTTCCTTCC TGGGTA, R: CTAGAGCATTTGCGGTGCA. The expected products are 557 nt for CXCR3, 267 nt for IFN-γ, and 350 nt for β-actin. To determine IFN-γ expression, bands were normalized with β-actin groups, and intensities were compared to determine fold of changes in gene expression (AlphaEase Fluor Chem 9900, Alpha Innotech, San Leandro, CA).

NO assay: The generation of nitrite in MΦ cultures was assessed by the Griess reaction, using the Nitrate/Nitrite Colorimetric Assay Kit (Caymann Chemical, Ann Arbor, MI). For each assay, MΦs (3 x 10^5 cells/ml) in 24-well plates were infected with 2.4 x 10^6 promastigotes (at 8:1 parasite-to-cell ratio) for 48 h. Culture supernatants were incubated with the Griess reagent (1:1 v/v) for 10 min at room temperature. The absorbance was measured at 540 nm, and nitrite concentration was determined using a standard curve of sodium nitrite and expressed as μM. In each experiment, supernatants from untreated, uninfected cells were included as negative controls.

Protein cytokine array: In the case of *in vitro* assays, BM-MΦ (1 x 10^6 cells/well) in 6-well plates were treated with 100 ng/ml of CXCL10 for 4 h and then infected with 8 x 10^6 *L. amazonensis* promastigotes (8:1) for 48 h. In the case of *in vivo* assays, infected mice were sacrificed at 3 and 6 wk post-infection to collect draining LN cells. Cells were pooled from 5 mice per group and cultured (5 x 10^6/well) in 6-well plates in the presence or absence of parasite lysate (1 x 10^7 parasite equivalents) for 48 h. The abundance and profile of cytokine production in supernatants were assessed using a Mouse Cytokine Antibody Array 2.1 (RayBioTech, Atlanta, GA). Briefly, membranes were sequentially incubated with blocking buffer and culture supernatants for 2 h, a cocktail of biotin-conjugated anti-cytokine Abs for 2 h, and then with HRP-conjugated streptavidin (1:1000) for 1 h. Detection was accomplished through the manufacturer's detection kit, and membranes were subsequently exposed to Kodak X-omat AR films (Fisher, Philadelphia, PA). Each membrane contained positive and negative controls and allowed the simultaneous detection of more than 20 molecules (two spots per molecule). The

intensity of positive controls was used to normalize membranes, and duplicate dots for each molecule were averaged for comparison (AlphaEase Fluor Chem 9900, Alpha Innotech, San Leandro, CA).

Data analysis: To evaluate the statistical significance among different groups, for parametric comparisons, a one-way ANOVA was used with a Tukey's Multiple Comparison test, while a Kruskal-Wallis test with a Dunn's Multiple Comparison post-test was used for non-parametric comparisons were performed using GraphPad Prism version 4.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com. Statistically significant values are referred to as * ($p < 0.05$) , ** *($p < 0.01$)* and *** ($p < 0.001$), respectively.

RESULTS

EXPRESSION OF CXCR3 ON MURINE BM-MΦS.

Although murine BM-MΦs are known to express various chemokine receptors, such as CCR2, the receptor for CCL2, it is unclear whether these cells also express CXCR3 either constitutively or conditionally. To address this issue, we examined CXCR3 expression in BM-MΦs of BALB/c mice and used LPS/IFN-γ-treated splenocytes as positive controls for CXCR3 induction. RT-PCR analyses indicated the presence of CXCR3 mRNA in unstimulated MΦs (Fig. 3.1A). To confirm the surface expression of CXCR3 on BM-MΦs, we performed FACS analysis and observed a small population (~10%) of double-positive cells for both CXCR3 and the pan-MΦ marker

F4/80 (Fig. 3.1B), suggesting the responsiveness of these cells to CXCL10. However, MΦs stimulated with LPS/IFN-γ did not significantly upregulate CXCR3 expression unlike in splenocytes (data not shown), which suggested that CXCR3 regulation on MΦs may differ from that observed in T cells (175).

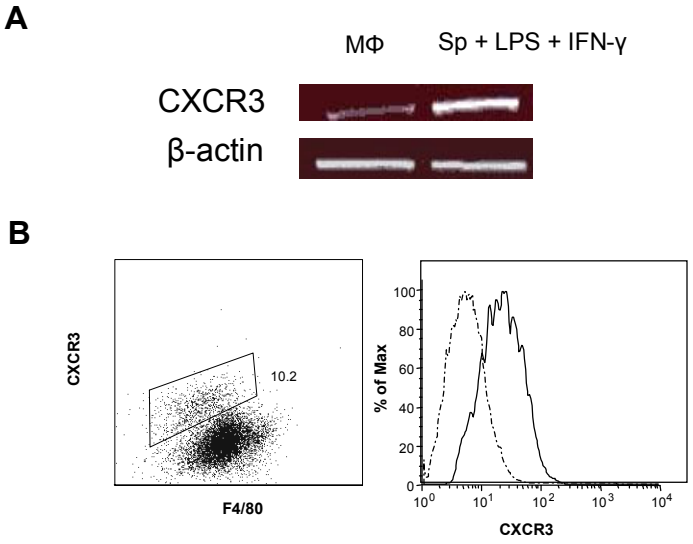


Figure 3.1 CXCR3 is expressed on murine BM-MΦs. BM-MΦs of BALB/c mice were seeded in 6-well plates (2.5×10^6 cells/well) and allowed to rest for 24 h. (A) Total RNA (100 ng) was extracted for RT-PCR analyses of CXCR3 and β-actin transcripts. Splenocytes from BALB/c mice were treated with LPS/IFN-γ and used as positive controls. (B) MΦs were collected and stained for surface expression of F4/80 and CXCR3 molecules. For flow cytometry, the single gate was set up according to isotype control, and the number represents the percentage of cells within the gate. Staining profiles of the isotype controls (dashed lines) versus the anti-CXCR3 group (solid lines) were included in the histogram overlay representing the shift in fluorescence.

TREATMENT WITH CXCL10 OR CCL2 PROMOTES REDUCTION OF *L. AMAZONENSIS* PROMASTIGOTE AND AMASTIGOTE INFECTION *IN VITRO*.

It has been reported that treatment of murine MΦs (158) or human monocytes (176) with recombinant CCR2 or CCR3 significantly reduces *L. donovani* and *L. infantum* parasite burdens through the induction of NO. To test whether this leishmanicidal activity can be induced in *L. amazonensis* infection, we evaluated the efficacy of exogenous CXCL10 (1-200 ng/ml) in parasite killing. At 48 h post-infection, parasite burden decreased with increasing amounts of CXCL10 and CCL2, indicating a

dose-dependent effect (data not shown). Since significant reduction in parasite burden was achieved for both tested chemokines at 100 ng/ml, this concentration was used for the subsequent studies reported herein.

As shown in Fig. 3.2, there was a 70% reduction in parasite loads in BALB/c MΦs (open bars) treated with CXCL10 in comparison to the infection controls. Under the same conditions, cells treated with CCL2 showed a 53% reduction in parasite load at 48 h, compared to the untreated controls, which was consistent with previous reports (158, 176). From these results, CXCL10 administration appeared to be more efficacious in eliminating intracellular parasites than CCL2 treatment, while the latter group was comparable to LPS/IFN-γ treatment. In addition, BM-MΦs generated from either B6 (black bars) or C3H mice (grey bars) demonstrated similar trends of parasite elimination when treated with CXCL10 (Fig. 3.2), suggesting that this treatment is not restricted to a single mouse genetic background. Because parasite reduction was observed in C3H mice, which are known to be hyporesponsive to LPS, this rules out the possibility that endotoxin contamination acted upon BM-MΦ activation during chemokine treatment.

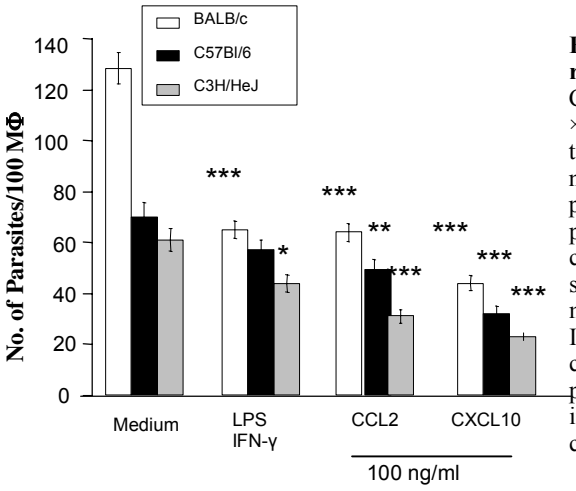


Figure 3.2 Pre-treatment with CXCL10 and CCL2 reduces parasite burden in MΦs. BM-MΦs of BALB/c, C57Bl/6 or C3H/HeJ mice were seeded in 24-well plates (3×10^5 cells/coverslip/well). Cells were left untreated or treated with either CXCL10 (100 ng/ml), CCL2 (100 ng/ml), or LPS (20 ng/ml) plus IFN-γ (20 ng/ml) for 4 h prior to infection with 2.4×10^6 stationary-phase promastigotes (at 8:1 parasite-to-cell ratio). All groups of cells were subsequently stained for parasites, using pooled sera from infected mice and FITC-conjugated, goat-anti mouse IgG. The nuclei of the cells were stained with DAPI. Images from 10 random fields per condition were taken for counting parasite loads. Data are presented as number of parasites per 100 MΦs, and shown is mean ± SD of three independent experiments (representing 30 fields per condition). (* $p < 0.05$; ** $p < 0.01$; $p < 0.001$).

Our group and others have reported that host cells respond differentially when treated with IFN-γ and subsequently infected with either *L. amazonensis* promastigotes or amastigotes. Specifically, IFN-γ-treated cells carry out NO-mediated killing of promastigotes (177), but promote growth of amastigotes (31). To determine whether CXCL10 treatment is effective in eliminating amastigote infection, we assessed parasite loads at 48 h post-infection. As shown in Fig. 3.3, parasite loads in CXCL10-treated cells (1.1×10^5 parasites/well) were not only significantly lower than those in the untreated control (7.7×10^5 parasites/well) ($p < 0.001$), but also significantly lower than those observed in CCL2-treated cells (3.0×10^5 parasites/well) ($p < 0.05$). Similar results were observed when MΦs of B6 mice were treated with CXCL10 or CCL2 (data not shown). Together, these results indicate the efficiency of CXCL10 administration in controlling infection initiated by both *L. amazonensis* promastigotes and amastigotes.

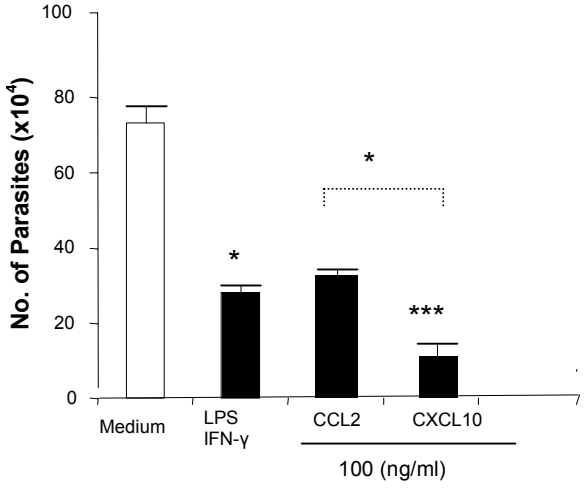


Figure 3.3 Pre-treatment with CXCL10 and CCL2 promotes the reduction of *L. amazonensis* amastigotes. BM-MΦs of BALB/c mice in 24-well plates (3×10^5/well) were left untreated (open bar) or treated (solid bars) with 100 ng/ml of CXCL10 or CCL2 for 4 h prior to infection with 6×10^5 tissue-derived amastigotes (at a 2:1 parasite-to-cell ratio). At 48 h post-infection, cells were lysed with 0.01% SDS, and parasites per well were counted with a hemocytometer (triplicate wells per condition). Data are presented as number of parasites per well, and shown is the mean ± SD of three independent experiments (resembling 9 wells per condition). Each group was compared to untreated controls (* $p < 0.05$; ** $p < 0.01$; ***$p < 0.001$).

THE INVOLVEMENT OF NO AND TH1 CYTOKINES IN CXCL10-MEDIATED PARASITE REDUCTION WITHIN MΦS.

Th1 cytokine-activated MΦs are known to kill intracellular parasites through the

production of toxic mediators such as NO, which is produced by iNOS, and the importance of NO in MΦ activation is well documented in animal models of *Leishmania* infection (158, 176). To address the role of NO in CXCL10-mediated parasite reduction, we measured the amount of nitrite in supernatants of stimulated, *L. amazonensis*-infected MΦs. Nitrite production in medium control cells was negligible. In the absence of parasites, treatment of cells with CXCL10, CCL2, or LPS/IFN-γ resulted in highly significant increases in NO production compared to that seen in medium controls ($p < 0.001$) (Fig. 3.4A).

Although cells treated with stimuli and then infected with parasites also presented significant amounts of NO, its levels were significantly lower in these cells than in those treated with CXCL10, CCL2, and LPS/IFN-γ in the absence of promastigotes ($p < 0.001$). This observation further substantiates the parasite-mediated inhibition of nitrite production as seen in previous studies (178). Interestingly, CXCL10 did not stimulate nitrite production in MΦs to a level comparable to those observed in LPS/IFN-γ-and CCL2-treated groups ($p < 0.01$), implying the involvement of other mechanisms in parasite killing in CXCL10 treatment.

To further examine the involvement of NO, we treated $iNOS^{null}$ B6 MΦs with CXCL10 prior to infection. As shown in Fig. 3.4B, there was a significant reduction of parasite loads in both wild-type and $iNOS^{null}$ MΦs treated with CXCL10, when the latter were compared to their relevant infection controls (Fig. 3.4B) ($p < 0.05$). This is in stark contrast to groups treated with CCL2, which resulted in significant increases in parasite

load in iNOS^{null} MΦs versus the findings following treatment of wild-type MΦs. This suggests that while NO is an important anti-*Leishmania* effector and is necessary for CCL2-mediated reduction of parasite burden in MΦs, it is not as crucial for CXCL10 and LPS/IFN-γ treatment, despite the high NO production.

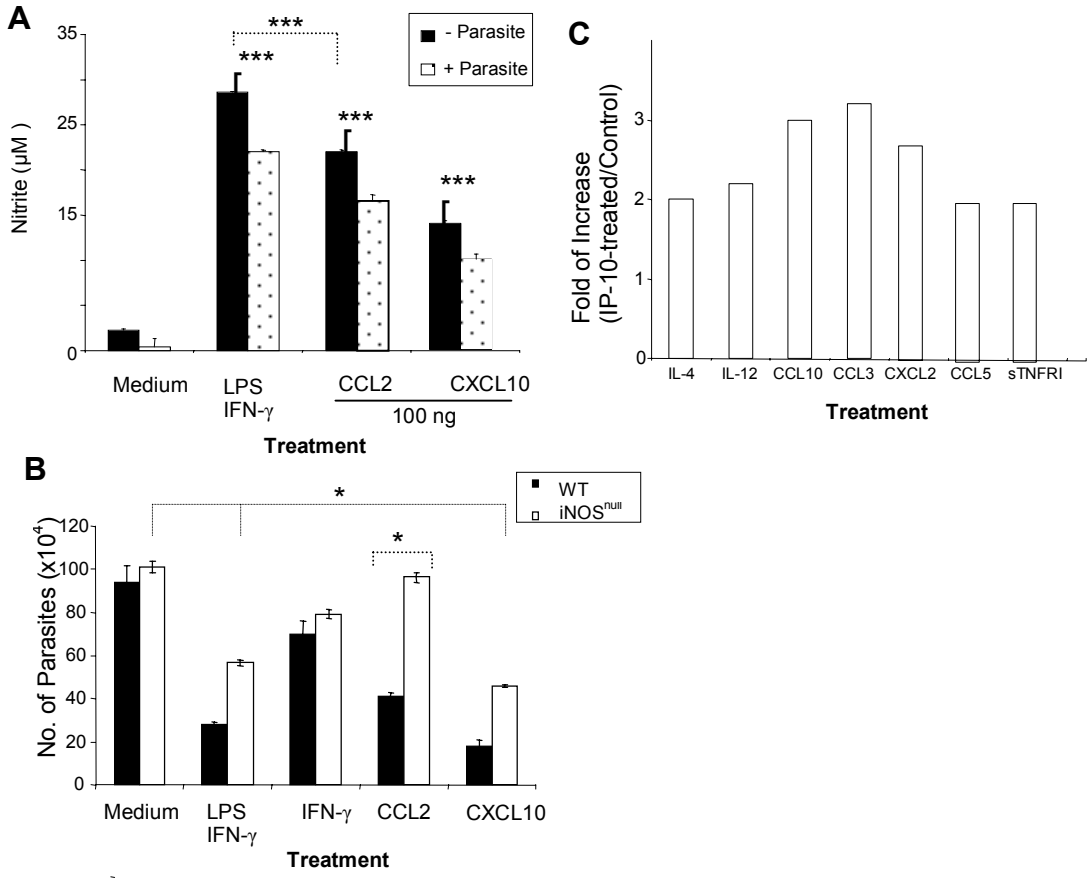


Figure 3.4 Nitric oxide and pro-inflammatory chemokines contribute to CXCL10- and CCL2-mediated parasite killing. (A) BM-MΦs of B6 mice (3×10^5 cells/well) in 24-well plates were left untreated or treated with indicated stimuli as in Fig 2 for 4 h prior to infection with 2.4×10^6 stationary-phase promastigotes (at 8:1 parasite-to-cell ratio). At 48 h post-infection, supernatants from uninfected (solid bars) and infected groups (dotted bars) were collected for measurement of nitrite via the Griess reagent. (B) BM-MΦs were generated from wild-type B6 mice (solid bars) or iNOS^{null} B6 mice (open bars), and treated with 100 ng/ml of CXCL10, CCL2 or IFN-γ for 4 h prior to infection with *L. amazonensis* lesion-derived amastigotes, as described in Fig 3.2. Cells treated with LPS (20 ng/ml) plus IFN-γ (20 ng/ml) served as positive controls. At 48 h post-infection, cells were treated with 0.01% SDS to release intracellular parasites, and parasite numbers per well were counted. Data are presented as number of parasites per well and expressed as mean ± SD for triplicate wells per condition. The iNOS^{null} groups were compared with wild-type counterparts (* $p < 0.05$; ** $p < 0.01$). Shown is a representative of three independent repeats.(C) BM-MΦs of BALB/c mice were seeded in 6-well plates (1×10^6 cells/well) and infected with 8×10^6 stationary-phase promastigotes (8:1 parasite-to-cell ratio). At 48 h post-infection, cell-free supernatants were collected for the measurement of cytokine profiles via protein cytokine arrays. The intensity of protein spots in the CXCL10-treated group was compared with the corresponding spots in the untreated control, and data are presented as fold of increase above the infection controls. Shown are the results for those molecules that displayed a ≥ 2-fold increase over the infection control, and are representative of three independent repeats.

In addition to the production of reactive nitrogen species, MΦs can also deliver a wide array of anti-microbial proteins via the release of inflammatory cytokines and chemokines (179). To examine the possible contribution of these molecules, we measured the profile of cytokines and chemokines by protein cytokine arrays. In comparison to the findings in infection controls, supernatants of CXCL10-treated, *L. amazonensis*-infected cells generated about a 3-fold increase in the production of CCL3, CXCL2 and CCL12, as well as a 2-fold increase in IL-4, IL-12, CCL5, and sTNFRI (soluble TNF receptor 1) (Fig. 3.4C). Production of other molecules was either unchanged (e.g., CCL2) or at a level below a 2-fold increase (e.g., CCL20). It appears that CXCL10 treatment can trigger the production of a selected set of inflammatory cytokines/chemokines in MΦ, suggesting that aside from NO elicitation, these molecules could potentially aid in the elimination of *L. amazonensis* parasites in MΦs.

LOCAL INJECTION OF CXCL10 DELAYS LESION DEVELOPMENT IN SUSCEPTIBLE B6 MICE BY STIMULATING THE PRODUCTION OF MULTIPLE EFFECTOR MOLECULES.

To define the *in vivo* function of CXCL10, we infected two groups of B6 mice with 1×10^5 *L. amazonensis* metacyclic promastigotes. In comparison to the PBS-injected controls, CXCL10-treated mice demonstrated a delay in disease onset, developed significantly smaller lesions at 6 to 10 wk post-infection (Fig. 3.5A), and contained significantly lower parasite loads in foot tissues at 10 wks (~2 logs, $p < 0.05$). Interestingly, we observed that IFN-γ expression at the site of infection in CXCL10-treated mice was 2.5-fold higher than that in infection controls (Figure 5B).

Since CXCL10 treatment significantly reduced parasite burden, we next examined the possible mechanisms underlying this protection. At 3 and 6 wk post-infection, DLN cells from *L. amazonensis*-infected, CXCL10-treated and PBS-injected mice were re-stimulated *in vitro* with *Leishmania* lysates for 48 h. Culture supernatants were analyzed for cytokine profiles using protein cytokine arrays.

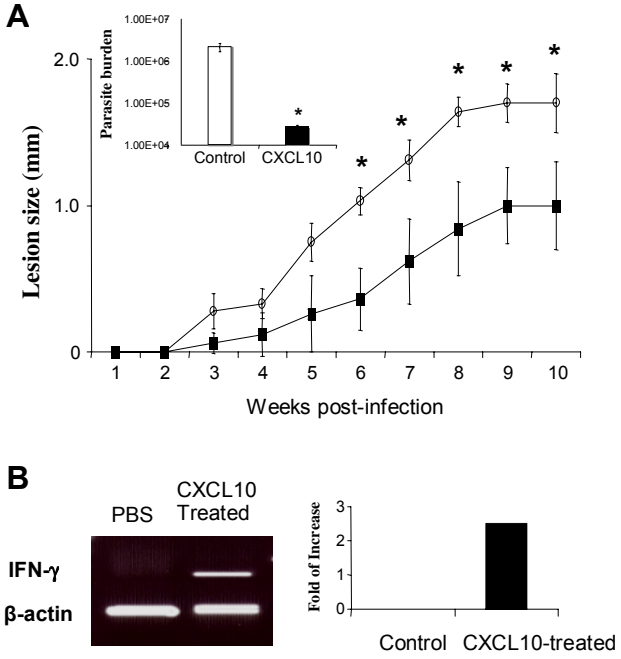


Figure 3.5 Local administration of CXCL10 delays lesion development in susceptible B6 mice. (A) B6 mice (5 per group) were infected s.c. with 2 × 10^5 *L. amazonensis* metacyclic promastigotes and treated s.c. with CXCL10 (100 ng in 5 μl) or PBS on days 1, 3 and 7 of infection. Lesion size (in mm) was monitored weekly, and shown is the mean ± SD for each group (the infected foot measurement minus the control foot measurement for each mouse). Results are representative of three independent experiments. Parasite burden per foot was determined at 10 wk post-infection and shown as the mean for each group. * ($p < 0.05$) indicates treated groups in comparison to the PBS controls. (B) At 10 wk post-infection, PBS- and CXCL10 treated mice were sacrificed, and total RNA was extracted from the infected foot tissues. RT-PCR analysis was performed to determine expression changes in IFN-γ. Band intensities were analyzed via spot densitometry and expression fold changes were determined after normalization with β-actin.

In comparison to the untreated controls at 3 wk post-infection, there was a 3-fold increase in the production of IL-2 and IL-3, as well as a 2-fold increase in IFN-γ, in CXCL10-treated groups (Fig. 3.6A). Except for a slight increase in IL-12 and CCL5 and a decrease in CXCL2 in the CXCL10-treated group, there were no major changes for other tested cytokines/chemokines in the arrays.

At 6 wk, however, CXCL10-treated mice displayed a unique, Th1 cytokine

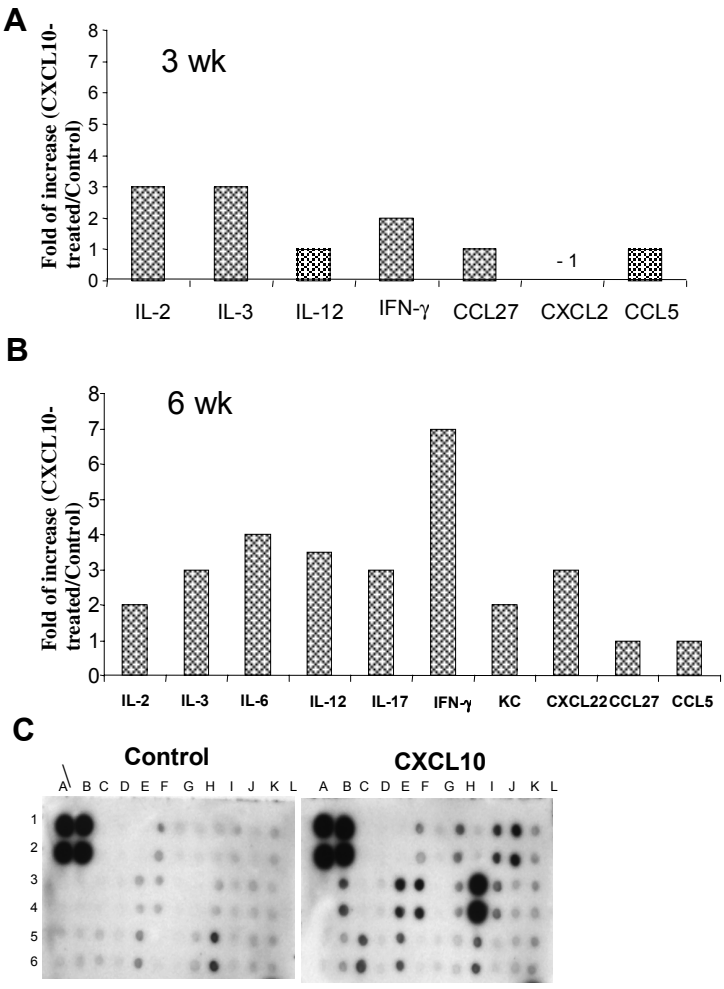


Figure 3.6 Local administration of CXCL10 triggers the production of multiple, Th1-favored cytokines and chemokines. B6 mice (5 per group) were infected and treated as in Fig. 5. At 3 wks (A) and 6 wks post-infection (B), LN cells (5×10^6/ml/well) were collected from CXCL10-treated groups or PBS controls, pooled from 5 mice, and stimulated with parasite antigen (1×10^7 parasite equivalents) for 48 h. Cell-free supernatants were collected for the measurement of cytokine profiles via protein cytokine arrays. The intensity of protein spots in the CXCL10-treated group was compared with the corresponding spots in the infection control, and data are presented as fold of increase above the infection controls. Data shown in A and B represent those molecules with > 2-fold of increase than the infection control at 3 and 6 wk post-infection, respectively. Example membranes from infection control and CXCL10 groups at 6 wk post-infection are shown in C.

profile in comparison to that in infection controls: a 7-fold increase in IFN-γ production, correlating with our RT-PCR finding at the lesion site (Fig. 3.5B), as well as a 3- to 4-fold increase in the production of IL-3, IL-6, IL-12, IL-17 and CXCL2 (Fig. 6B). Evidently, CXCL10 treatment triggered the up-regulation of a selected set of cytokines and chemokines at 6 wk of infection because some molecules (e.g., IL-4, CCL3, and CCL5) showed no or marginal changes in comparison to those found in the controls (Fig. 3.6 C). Overall, these results suggest that exogenous CXCL10 stimulates effector cells to

produce Th1 and other pro-inflammatory cytokines, which contribute to the partial control of *L. amazonensis* infection *in vivo*.

DISCUSSION

The mechanism underlying non-healing cutaneous leishmaniasis associated with *L. amazonensis* infection remains largely unresolved. One possibility is that *L. amazonensis* parasites evade the host immune response by inhibiting the early inflammatory cytokine and chemokine responses, thereby preventing the expansion of antigen-specific Th1 cells even in the absence of Th2 dominance (127). Since CXCL10 is a CXC chemokine known to favor the recruitment and activation of Th1-polarized cells (180), we tested the prospect that exogenous CXCL10 would significantly reduce parasite loads, and that CXCL10 injection at early stages of infection would skew the immune responses and influence the outcome of *L. amazonensis* infection *in vivo*.

In the present study, we have provided evidence that exogenous CXCL10 markedly enhances the responsiveness of MΦs to *L. amazonensis* infection. First, CXCL10 treatment significantly reduces infection prevalences and parasite loads, regardless of the initiation of infection by promastigotes (Fig. 3.2) or lesion-derived amastigotes (Fig. 3.3). The effect of CXCL10 treatment is markedly different from that of IFN-γ treatment (31), and the leishmanicidal activity of CXCL10 is more potent than LPS/IFN-γ or CCL2 (Figs. 3.2-3.4), suggesting a therapeutic potential of this chemokine in the control of *Leishmania* infection.

Although a wealth of information exists concerning the role of CXCL10 on Th cell polarization (150, 181, 182) and the requirement for CXCR3 in the migration of activated $CD4^+$ and $CD8^+$ T cells to inflamed dermal sites (170), especially those induced by *L. major* infection (180), studies aimed at examining the role of CXCL10 in *L. major* infection have yielded ambiguous results. For example, an early and strong induction of the CXCL10 gene correlated nicely with the healing phenotype in *L. major*-resistant B6 mice (171, 183). A single injection of CXCL10 into *L. major*-susceptible BALB/c mice, however, led to enhanced lesion development (169). Furthermore, it was not clear in the latter report whether heightened footpad swelling was due to increased parasite loads and/or elevated local responses. In the present study, we have shown that three injections of CXCL10 at early stages of infection with *L. amazonensis* parasites resulted in a delayed onset of disease, reduced lesion sizes, and reduced parasite loads (Fig. 3.5), and that this enhanced resistance to *L. amazonensis* infection in CXCL10-treated mice correlated with a partial reversal of immune non-responsiveness associated with *L. amazonensis* infection and development of a Th1-biased response (Fig. 3.6) (127). The discrepancy between the effects of CXCL10 on different models of cutaneous leishmaniasis may not be surprising, given the known differences in virulence factors of Old and New World species of *Leishmania* responsible for cutaneous diseases (128), as well as marked differences in the host immune response to *L. amazonensis* and *L. major* infection (30, 126, 127, 183).

IL-4 production is often linked to the susceptibility of BALB/c mice to *L. major* infection; however, an IL-4-independent impairment of the host response is a hallmark

for non-healing disease caused by *L. amazonensis* infection (126, 127). We speculate that, when given during the first few days of *L. amazonensis* infection, exogenous CXCL10 acts on multiple cell types in the innate arm of host defense, triggering a positive loop for Th1 responses that are necessary for the control of disease progression. At the MΦ level, we have shown that CXCL10 treatment stimulates the production of IL-12, CCL12, CCL3, and CXCL2 (Fig. 3.4), which may, in turn, augment the activation of NO production and other leishmanicidal mechanisms for further reducing loads. At the tissue level, exogenous CXCL10 may act on multiple cell types, including MΦs, NK cells, $CD4^+$ and $CD8^+$ T cells, resulting in an enhanced innate immunity and parasite-specific Th1 responses in the local tissue (Figs. 3.5 and 3.6). It has been reported that s.c. injection with 1 μg of recombinant CXCL10 in the footpad of BALB/c mice 2 h prior to infection with *L. major* resulted in markedly enhanced NK cell cytotoxic activities (171). Evidence from a visceral leishmaniasis model also supports CXCL10 activation of IFN-γ-producing cells and the innate response to *L. donovani* (168). Studies are ongoing to further examine the effect(s) of CXCL10 on DCs, $CD4^+$ and $CD8^+$ T cells, and NK cells that may contribute to enhanced resistance in CXCL10-treated mice.

Of note, we observed a 3-fold increase in IL-17 expression in CXCL10-treated mice at 6 wk of infection (Fig. 3.6). IL-17 is the prototypical member of a new cytokine family that is involved in the proliferation, maturation, and chemotaxis of neutrophils (184, 185), and in combating intracellular microorganisms (186). IL-17 has pleiotropic activities, including the induction or amplification of the effects of TNF-α, IFN-γ, IL-1, IL-6, IL-8, CXCL10, CXCL11 and CCL2 expression and recruitment of neutrophils

(184, 187). Since IL-17 also acts on T cells as a co-stimulatory factor (186), more investigation is needed to determine the role of IL-17 in *L. amazonensis* and other *Leishmania* infections.

Although CXCL10-treated mice mounted appreciable levels of innate responses and antigen-specific Th1 responses, these mice remained susceptible to *L. amazonensis* infection. Several possibilities may account for these observations. First, the half-life of recombinant CXCL10 in vivo may not be sufficient to provide a long-lasting effect. The use of a CXCL10/Ig fusion protein or CXCL10-expressing plasmid may partially overcome this problem. In this regard, it has been reported that injection of CXCL10-encoding expression vector can redirect and promote antigen-specific, Th1-biased responses, suggesting its potential as an immunotherapy (188). Co-administration of DNA-encoding CXCL10 and IL-12 has been shown to markedly enhance the anti-tumor efficiency of the individual DNA (189). Second, *L. amazonensis* parasites may have intrinsic, undefined mechanisms to evade cytokine- and chemokine-mediated immune responses (127). It has been suggested that *L. donovani* parasites secrete a chemotactic factor that preferentially inhibits CXCL10 production from neutrophils, which could prevent NK cell activation (172). Finally, local injection of CXCL10 may also trigger the production of multi-functionary cytokines such as IL-3, IL-6 and CXCL2 (Fig. 3.6). IL-3 and CXCL2 have been reported to enhance parasite loads and lesion development in mice infected with *L. major* parasites (190-192); yet, their roles in *L. amazonensis* infection are currently unclear. Although recombinant IL-6 has been shown to down-modulate cytokine production of human MΦs through oxygen-dependent mechanisms and to

reduce host responses to *Leishmania* parasites (192), no significant change has been observed in *L. major*-infected, IL-6^{null} BALB/c mice, which makes the role of IL-6 in immunity to *Leishmania* parasites questionable (193). Regardless of the mechanisms, it will be interesting to further examine the efficacy of the prolonged use of CXCL10, alone or in combination with other cytokines, in the control of *L. amazonensis* infection, especially an established infection.

In summary, we have shown the ability of CXCL10 to activate MΦs for the production of pro-inflammatory mediators and the control of *L. amazonensis* infection *in vitro*. To the best of our knowledge, this is the first report examining this effect on MΦs the context of pathogen infection. In addition to previous studies examining chemokines as potential targets for a potential anti-*Leishmania* therapy (169, 194), we have demonstrated that direct injection of CXCL10 can overcome the deficient immune response associated with *L. amazonensis* infection, thereby skewing the host's cytokine profile towards a more vigorous Th1 response. While injection of recombinant CXCL10 alone is insufficient to provide long-lasting protective immunity against *L. amazonensis* infection, this study provides important insight into mechanisms underlying the pathogenesis associated with non-healing, New World cutaneous leishmaniasis and lays the groundwork for further study of treatment regiment.

CHAPTER 4: EFFECTS OF CXCL10 ON DENDRITIC CELL AND $CD4^+$ T CELL FUNCTIONS DURING *L. AMAZONENSIS* INFECTION

INTRODUCTION

Leishmania are obligate intracellular parasites that replicate within mammalian cells in the MΦ lineage, resulting in an array of clinical syndromes categorized by their disease manifestations (128). Immune regulation of host responses to *Leishmania* has been extensively investigated in murine models of leishmaniasis. Specifically, resistance to *L. major* in C57BL/6 (B6) or C3H mice is clearly linked to a dominant Th1 response (164). Susceptibility of BALB/c mice to *L. major* is, in most cases, reliant upon early production of IL-4 (164), which promotes lesion development during the early stages of infection. Meanwhile, long-term persistence of *L. major* in the resistant strains of mice appears to be mediated by IL-10 production (64).

Unlike infections with *L. major*, most inbred mouse strains are susceptible to *L. amazonensis* infection. Studies from our group and others have indicated a Th1/Th2-mixed response, rather than Th2 dominance, as the central characteristic for non-healing lesions in *L. amazonensis*-infected hosts (124, 129). In addition, B cells and circulating antibodies may also contribute to *L. amazonensis* pathogenesis (133) via multiple mechanisms, including antibody-mediated IL-10 production (120). Decreased production of IL-12, coupled with a diminished expression of the IL-12Rβ_2 chain on Th1 cells in *L. amazonensis*-infected mice suggest that an IL-4-independent mechanism is responsible for reduced IL-12 responsiveness and consequently, impaired Th1 response in *L. amazonensis* -infected hosts (166).

Attempts at boosting protective immunity against *L. amazonensis* has been met with limited success. Our laboratory and others have explored the potential of DNA- and parasite antigen-based vaccines (195), adoptive transfer of *L. amazonensis*-specific Th1 $CD4^+$ T cell line (31, 127) or antigen-pulsed DCs (196) in the control of *L. amazonensis* infection in mice. Although each study demonstrated an increase in IFN-γ production, *L. amazonensis* parasite burden was not completely eliminated and clinical syndromes eventually returned. Recombinant IL-12 (166) or IL-1α (167) when administered prior to *L. major* infection, made otherwise susceptible BALB/c mice resistant; however, exogenous IL-12 (125) or IL-1β (Xin and Soong, in press) did not ameliorate *L. amazonensis* disease progression in *L. amazonensis*-infected mice. These studies indicate that other factors should be explored as potential therapeutic interventions for the control of non-healing cutaneous leishmaniasis.

Chemokines play an important role in the proper development and functional aspects of leukocytes, as well as being crucial for the defense against pathogens. CXCL10/IP-10 (IFN-γ-inducible protein 10) binds with high affinity to CXCR3 (168), a receptor known to be expressed on several types of cells in the hematopoietic lineage, including activated and memory $CD4^+$ and $CD8^+$ T cells, NK cells, MΦs, and subsets of DCs (170). The therapeutic potential of chemokines in the control of *Leishmania* infections has been explored recently (158, 176). For example, CXCL10 was found to activate leishmanicidal activity in murine MΦ, resulting in a significant reduction in parasite burden *in vitro*, while local injection of CXCL10 markedly delayed disease development in susceptible B6 mice (197). This enhanced parasite killing was partially due to an increased production of multiple effector molecules, such as IFN-γ, IL-12 and

nitric oxide (NO) following CXCL10 treatment. However, it is unclear whether CXCL10 can directly promote DC activation and Th1 responses to *Leishmania* infection.

Since both DCs and $CD4^+$ T cells express CXCR3, the cognate receptor for CXCL10 (156, 198), we hypothesize that CXCL10 treatment will result in an enhanced expression of IL-12 in DCs and IFN-γ production in $CD4^+$ T cells, respectively. Here we showed that CXCL10 could induce IL-12p40 but reduce IL-10 production in bone marrow-derived murine DCs. However, *L. amazonensis*-infected DCs exhibited elevated IL-10, despite the presence of exogenous CXCL10. Elimination of endogenous IL-10 increased the responsiveness of DCs and T cells to CXCL10, as judged by enhanced IL-12 production in DCs and increased priming and expansion of Th1-like $CD4^+$ T cells. Furthermore, CXCL10 stimulated $CD4^+$ T cells to express the IL-12Rβ_2 chain and produce elevated IFN-γ. Collectively, these results indicate that exogenous CXCL10 triggers a Th1-favored, pro-inflammatory phenotype, which aids in the reduction of clinical syndromes caused by *L. amazonensis*. This study further highlights the potential utility of CXCL10 as an immune modulator for the treatment of non-healing cutaneous leishmaniasis.

MATERIALS AND METHODS

Mice: IL-10^{null} and wild-type C57BL/6 (B6) mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Mice were maintained under specific pathogen-free conditions and used at 6-10 weeks of age. All protocols were approved by the Animal Care and Use Committee of the University of Texas Medical Branch (Galveston, TX).

Parasites: *L. amazonensis* (MHOM/BR/77/LTB0016) parasites were used in all the experiments, and their infectivity was maintained by regular passage through BALB/c mice. Promastigotes were cultured in 20% FBS-supplemented (Hyclone, Logan, UT) Schneider's *Drosophila* media (Life Technologies, Rockville, MD), pH 7 at 23^{o}C. Amastigotes were cultured in the same medium, pH 5 at 32^{o}C.

BM-DC culture and parasite infection: BM-DCs derived from either from WT or IL-10^{null} mice were cultured *in vitro* in the presence of 20 ng/ml of murine granulocyte-macrophage colony-stimulating factor (eBioscience, San Diego, CA), according to our previous report (43). Briefly, at day 8 of culture, DCs were left untreated or pre-treated with recombinant mouse CXCL10 (200 ng/ml, Leinco Technologies, St. Louis, MO), or 20 ng/ml of LPS of *Salmonella enterica* serovar Typhimurium (Sigma, St. Louis, MO) plus 20 ng/ml of IFN-γ (Leinco Technologies) for 4 h. Cells were then infected with promastigotes at an 8:1 parasite-to-cell ratio at 33°C for 12 h, then at 37°C for another 12 h.

FACS analysis for DC surface markers and intracellular cytokines: The following specific mAbs were purchased from eBioscience unless stated otherwise: FITC-conjugated anti-CD45RB (C363.16A), PE-conjugated anti-CD83 (Michel-17), anti-IL-12p40 (C17.8), PE-Cy5-conjugated anti-CD11c (N418), as well as isotype control Abs, including FITC-conjugated rat IgG2a; PE-conjugated rat IgG2a, and IgG2b; PE-Cy5-conjugated Hamster IgG. All staining steps were performed on ice. For intracellular staining of IL-12p40, DCs were infected with parasites for 24 h, and 1 μl GolgiStop (BD Biosciences, San Jose, CA) was added 6 h before harvest. Cells were washed and blocked for nonspecific Ab binding and Fc receptors using purified anti-mouse CD16/32, hamster

IgG (Pierce, Rockford, IL) and 2% rat serum (Sigma) for 30 min. Cells were stained for CD11c, CD45RB and CD83. After surface staining, cells were fixed/permeabilized with a Cytofix/Cytoperm Kit (BD Biosciences) and then stained for IL-12p40. Appropriate isotype controls were included for both surface and intracellular staining. Cells were analyzed on a FACScan (BD Biosciences) using the FlowJo software (TreeStar, Ashland, OR).

Isolation and stimulation of $CD4^+$ T cells: $CD4^+$ T cells were purified from the spleen of naïve B6 mice, or from DLN of B6 mice infected with *La* for 8 to 10 wks, via negative selection using magnetic beads (Miltenyi Biotec, Auburn, CA). FACS analysis indicated ~95% $CD4^+$ purity. Isolated $CD4^+$ T cells were plates in 12-well plates at a concentration of 1×10^7/well. Cells were left untreated or treated with CXCL10 (200 ng/ml), or with Concanavalin A (Con A, 2 ng/ml; Sigma) for 24 h. Total RNA was then extracted via Tri reagent (Sigma).

RT-PCR analysis for cytokine expression: Total RNA (200 ng) were subjected to RT-PCR analysis, and annealing temperatures were at 58°C for β-actin and at 59°C for IL-12Rβ_2 and IFN-γ. Primer sequences (listed as 5' to 3') were as follows: IL-12Rβ_2 F: GGGGCTGCATCCTCCATTAC; R: AAGTGCTGTTTGCTGGATCTG; IFN-γ F: CATTGAAAGCCTAGAAAGTCTG; R: CTCATGGAATGCATCCTTTTTCG; β-actin F: CCAGCCTTCCTTCCTGGGTA; R: CTAGAGCATTTGCGGTGCA. The expected PCR products were 405 nt for IL-12Rβ_2, 267 nt for IFN-γ, and 350 nt for β-actin, respectively.

DC-T cell coculture and T cell proliferation assay: Purified $CD4^+$ T cells (2×10^5)

were cocultured with parasite-infected, mitomycin C-pretreated (30 min) DCs (2×10^4) in round-bottom, 96-well plates for 4 days in a total volume of 200 μl. Culture supernatants were harvested for cytokine detection. To determine $CD4^+$ T cell proliferation, 1 μCi of [^{3}H] thymidine was added 18 h before harvest. Incorporated radioactivity (cpm) was determined on a microplate scintillation and luminescence counter (Packard Instrument Company; Groove, IL).

Cytokine ELISA: Cytokine levels in the supernatants of infected DCs, T cells, as well as from DC-T cell coculture, were measured by ELISA specific for IL-12p40, IL-10 and IFN-γ (BD Biosciences). Detection limits were 16 pg/ml for IFN-γ, 4 pg/ml for IL-10, and 10 pg/ml for IL-12p40, respectively.

Data analysis: To evaluate the statistical significance among different groups, a one-way ANOVA with a Tukey's Multiple Comparison post-test were performed utilizing GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA). A difference in mean values was deemed significant when $p < 0.05$ or very significant when $p < 0.01$.

RESULTS

CXCL10 TREATMENT STIMULATES IL-12 BUT SUPPRESSES IL-10 PRODUCTION BY DCS.

CXCL10 can stimulate murine MΦs to produce several pro-inflammatory cytokines and chemokines, and intradermal injection reduced lesion development in susceptible C57BL/6 mice (197). Given the known expression of CXCR3 on DCs (198) and the critical roles of DCs in innate and \acquired immunity to *Leishmania* (43, 200),

we investigated the stimulatory effects of CXCL10 on BM-DCs. We first focused our study on CD45RB^{low}CD11c^{+} DCs, since this subset of cells are activated (expressing high levels of CD40 and CD83) and are the major producers for IL-12p40 in response to infection with *Leishmania* promastigotes (Xin and Soong, in press).

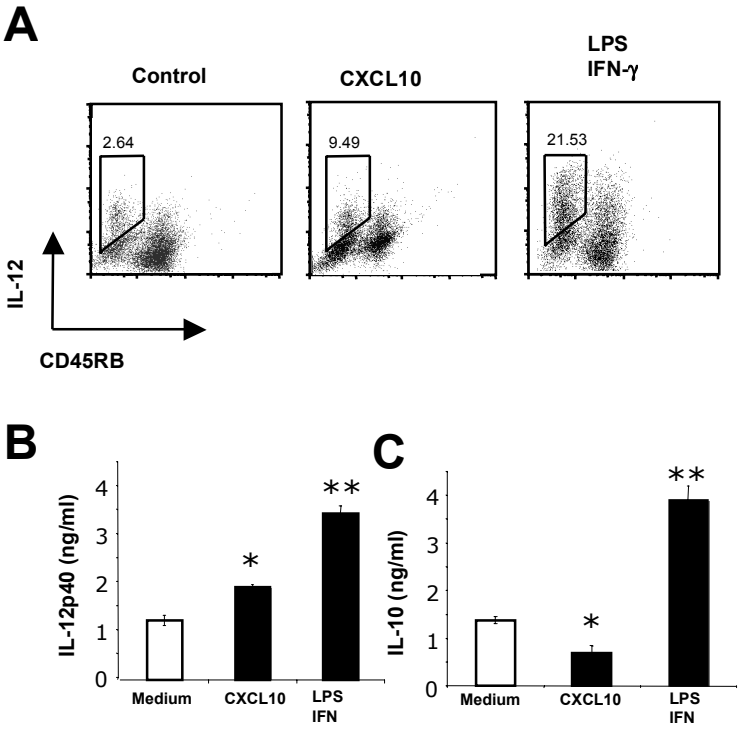


Figure 4.1 CXCL10 treatment stimulates IL-12 but suppresses IL-10 production. BM-DCs of B6 mice were seeded in 12-well plates (3 x 10^6 cells/well) and were subsequently treated with 200 ng/ml of CXCL10 or 20 ng/ml of LPS and IFN-γ for 24 h. (A) DCs were collected and stained for surface expression of CD11c and CD45RB molecules plus intracellular IL-12p40. Shown are percentages of positively stained cells gated on CD11c^{+} cells. Data represents five independent experiments with similar results. The levels of (B) IL-12p40 and (C) IL-10 in the supernatants were assayed by ELISA. Results are shown as mean ± SD from four independent experiments performed in duplicate. * ($p<0.05$) and ** ($p<0.01$) indicate statistically significant differences between the treated groups and medium controls.

As shown in Fig. 4.1A, following treatment with 200 ng/ml of recombinant CXCL10 for 24 h, ~9.5% of CD45^{low}, CD11c^{+} DCs were positive for intracellular IL-12p40, which represented a 3.5-fold increase in comparison to untreated cells (2.6%). As expected, DCs treated with LPS/IFN-γ displayed an 8-fold increase in IL-12-producing DCs compared to untreated cells. No IL-10-producing cells were detected in any groups by intracellular staining (data not shown). Using ELISA, we found a significant elevation of IL-12p40 production (Fig. 4.1B; $p < 0.05$), but reduced IL-10 secretion in CXCL10-treated DCs in comparison to medium controls (Fig. 4.1C; $p < 0.05$). These observations suggest that CXCL10 treatment polarizes DCs towards a pro-inflammatory, IL-12-

producing phenotype, which may provoke Th1 differentiation upon engagement with naïve T cells (199).

EXOGENOUS CXCL10 DOES NOT REVERSE THE IMMUNOMODULATORY EFFECT OF L. AMAZONENSIS ON IL-10.

Since *Leishmania* parasites can modulate DC activation and function (43, 200, 201), we next examined the effect of CXCL10 on DC maturation and activation during *L. amazonensis* infection. Promastigote-infected DCs (8:1 parasite-to-DC ratio) treated subsequently with CXCL10, showed enhanced activation, as judged by high frequencies of both $CD83^{+}CD45RB^{low}$ DCs (data not shown) and IL-12p40-producing cells (Fig. 4.2A). Exogenous CXCL10 also increased the frequency of IL-12p40-producing, $CD11c^{+}$,$CD45RB^{low}$ DC subset from 5.96% (parasite alone) to 10.5%. However, the percentage of IL-12-producing cells in *L. amazonensis*-infected, CXCL10-treated DCs decreased to 7.3%, a notable and consistent decline in comparison to CXCL10-treated DCs (Fig. 4.2A).

ELISA results further confirmed the observations from FACS staining (Fig. 4.2B), showing lower levels (1,085 pg/ml) of IL-12p40 in *L. amazonensis*-infected cells in contrast to *L. amazonensis*-infected, CXCL10-treated DCs (1,389 pg/ml; $p < 0.05$). As shown in Fig. 4.2C, *L. amazonensis*-infected DCs produced significantly higher levels of IL-10 (900 pg/ml) when compared to medium controls (500 pg/ml; $p < 0.05$). Of note, IL-10 production was significantly higher in *L. amazonensis*-infected, CXCL10-treated DCs (1,174 pg/ml, $p < 0.05$) than in DCs given parasites alone. *L. amazonensis*-infected CXCL10-treated DCs displayed the highest levels of IL-10 among all tested groups. These observations infer that despite the ability of CXCL10 to elicit Th1-biased

cytokines, *L. amazonensis* infection can modulate DC responsiveness, favoring the production of IL-10.

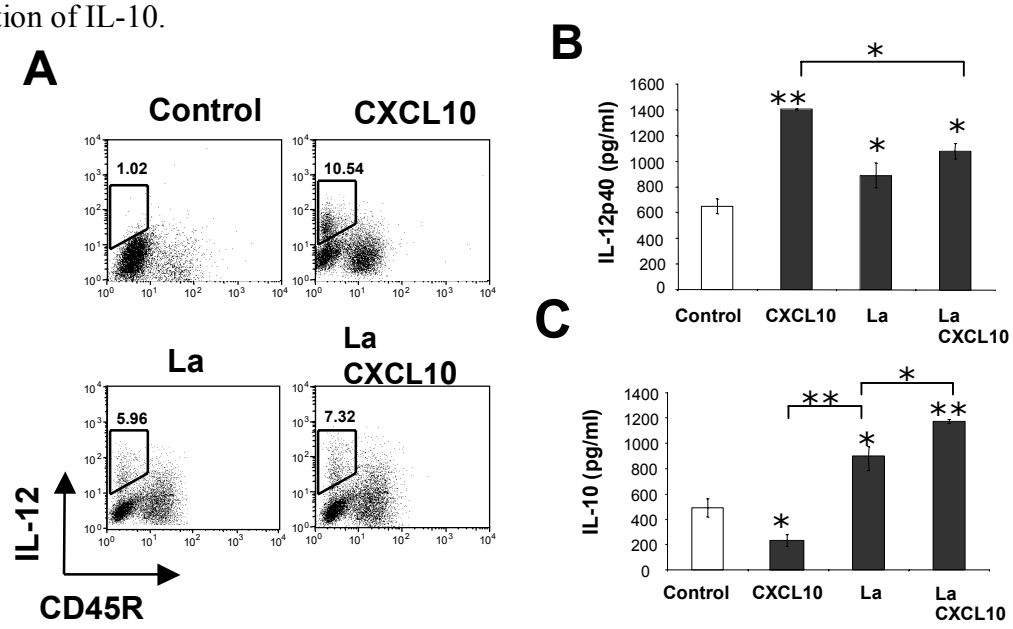


Figure 4.2 Exogenous CXCL10 does not reverse the immunomodulatory effect of *L. amazonensis* on IL-10. BM-DCs of B6 mice were seeded in 12-well plates (3 x 10^6 cells/well) and infected with 2.4 x 10^7 *L. amazonensis* promastigotes. At 4 h post-infection, cells were treated with 200 ng/ml CXCL10 for 24 h. (A) DCs were collected and stained for surface expression of CD11c and CD45RB molecules plus intracellular IL-12. Shown are percentages of positively stained cells gated on $CD11c^+$ cells. Data represents six independent experiments with similar results. The amount of IL-12p40 (B) and IL-10 (C) in the supernatants were assayed by ELISA. Results are shown as mean ± SD from five independent experiments performed in duplicate. * ($p<0.05$), ** ($p<0.01$) indicate statistically significant differences between the indicated groups.

IL-10^{NULL} DCS PRODUCE INCREASED IL-12 FOLLOWING CXCL10 TREATMENT.

IL-10 is a well-known immunosuppressive cytokine that is essential in down-modulating immune responses to numerous pathogens and preventing immune pathology (201). Indeed, DCs matured in the presence of IL-10 gave rise to regulatory DCs, which promoted T cell anergy and tolerance (202). *L. amazonensis* parasites have been shown to down-regulate IL-12 production in DCs and to limit DC APC function via multiple mechanisms, including, but not limited to IL-10 release (43, 126). To assess the involvement of IL-10 in CXCL10-medicated DC activation, we utilized BM-DCs from IL-10^{null} mice. Compared to WT DCs, IL-10^{null} DCs had an elevated baseline production of IL-12p40, as well as stimulation/infection-induced IL-12 secretion (Fig. 4.3A & B).

Consistently, we observed that in comparison to their WT counterparts, deletion of IL-10 resulted in approximately 3% and 7% increases in the frequencies of IL-12-producing DCs following treatment with CXCL10 or infection with parasites, respectively (Fig. 4.3A). Most strikingly, there was a near 11% increase in the frequencies of IL-12-producing cells in *L. amazonensis*-infected, CXCL10-treated IL-10^{null} DCs in comparison to their WT counterparts. Consistent with these FACS data, ELISA analysis (Fig. 4.3B) also showed significant increase in IL-12p40 production in all IL-10^{null} groups versus their WT DC counterparts ($p < 0.05$), as well as among IL-10^{null} DCs following treatment with CXCL10 and parasite infection ($p < 0.05$). Collectively, these data indicate that abrogation of endogenous IL-10 can markedly enhance CXCL10-mediated and *Leishmania* infection-mediated DC activation, via promoting IL-12 production.

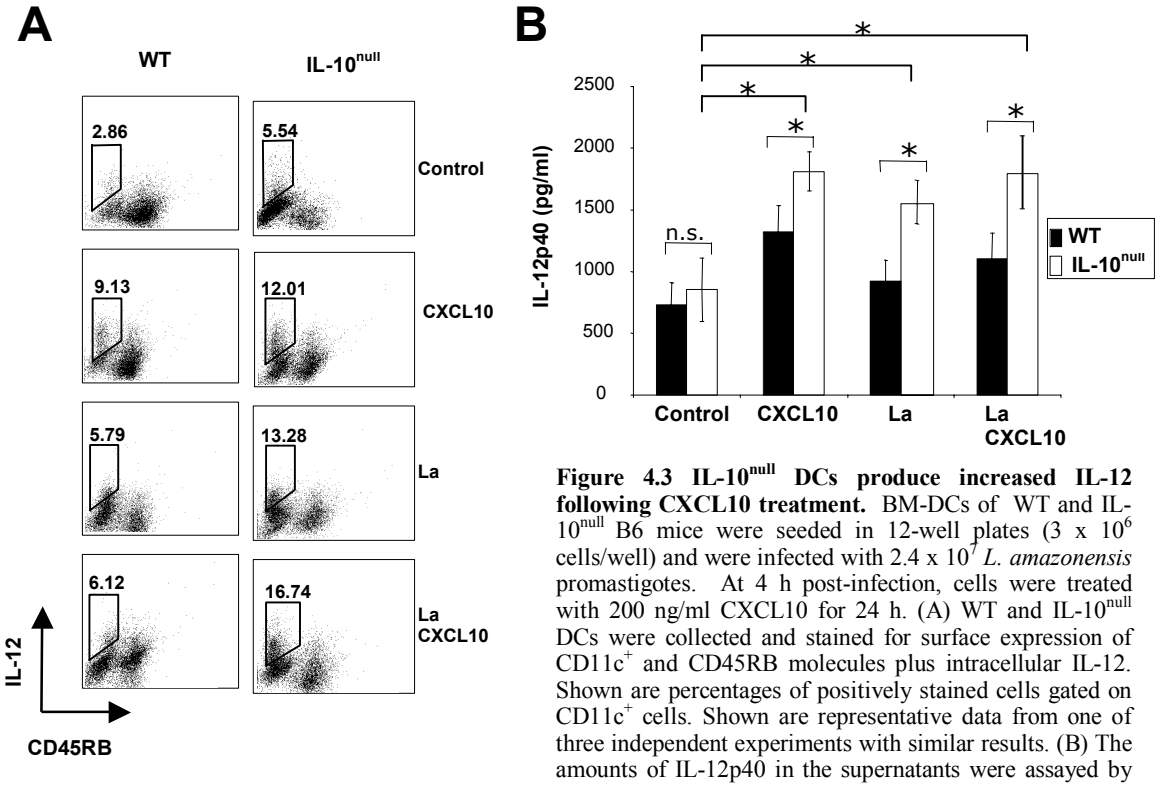


Figure 4.3 IL-10^{null} DCs produce increased IL-12 following CXCL10 treatment. BM-DCs of WT and IL-10^{null} B6 mice were seeded in 12-well plates (3 x 10^6 cells/well) and were infected with 2.4 x 10^7 *L. amazonensis* promastigotes. At 4 h post-infection, cells were treated with 200 ng/ml CXCL10 for 24 h. (A) WT and IL-10^{null} DCs were collected and stained for surface expression of CD11c$^+$ and CD45RB molecules plus intracellular IL-12. Shown are percentages of positively stained cells gated on CD11c$^+$ cells. Shown are representative data from one of three independent experiments with similar results. (B) The amounts of IL-12p40 in the supernatants were assayed by ELISA. Results are shown as mean ± SD from three independent experiments performed in duplicate. * ($p<0.05$) represents statistically significant differences between the indicated groups. n.s. indicates groups that were not statistically significant ($p>0.05$).

DIFFERENTIAL EFFECTS OF CXCL10 ON WT AND IL-10^{NULL} CD4^{+} T CELL ACTIVATION.

To determine the interplay of CXCL10 and IL-10 in DC APC function during *L. amazonensis* infection, we used an *in vitro* T cell priming assay. CD4^{+} T cells were purified from the spleen of naïve WT and IL-10^{null} B6 mice and co-incubated with mitomycin C-treated DCs that were previously infected with *L. amazonensis* and then treated with CXCL10 for 24 h. The proliferation of CD4^{+} responder T cells was measured after 4 days of co-culture. While CXCL10 treatment of DCs had no or marginal effect on CD4^{+} T cell proliferation (black bars), there was a significant increase in the proliferation of IL-10^{null} CD4^{+} T cells cocultured with CXCL10-treated IL-10^{null} DCs (open bars) (Fig. 4.4A, $p < 0.01$). Of note, proliferation of IL-10^{null} CD4^{+} T cell was substantially increased following incubation with *L. amazonensis*-infected, CXCL10-treated IL-10^{null} DCs compared to their WT analogue ($p < 0.01$), as well as with IL-10^{null} CD4^{+} T cells co-cultured with *L. amazonensis*-infected IL-10^{null} DCs (Fig. 4.4A, $p < 0.01$). These observations suggest that in the absence of IL-10, CXCL10 can promote the priming and expansion of *L. amazonensis*-specific CD4^{+} T cells.

Since activation of CD4^{+} T cells is responsible for both protective immunity and disease pathogenesis, depending on the magnitude of Th1-type responses (30, 125, 127, 129), we next determined whether CXCL10-treatment could increase the production of Th1-biased cytokines. As expected, we found that IL-10 secretion was significantly lower among all IL-10^{null} groups in comparison to their WT counterparts (Fig. 4.4B; $p < 0.05$). CD4^{+} T cells co-cultured with CXCL10-treated DCs produced less IL-10 as compared to the control ($p < 0.05$), suggesting that CXCL10 treatment can also suppress IL-10 release in CD4^{+} T cells, as well as in CXCL10-treated DCs (Fig. 4.2C).

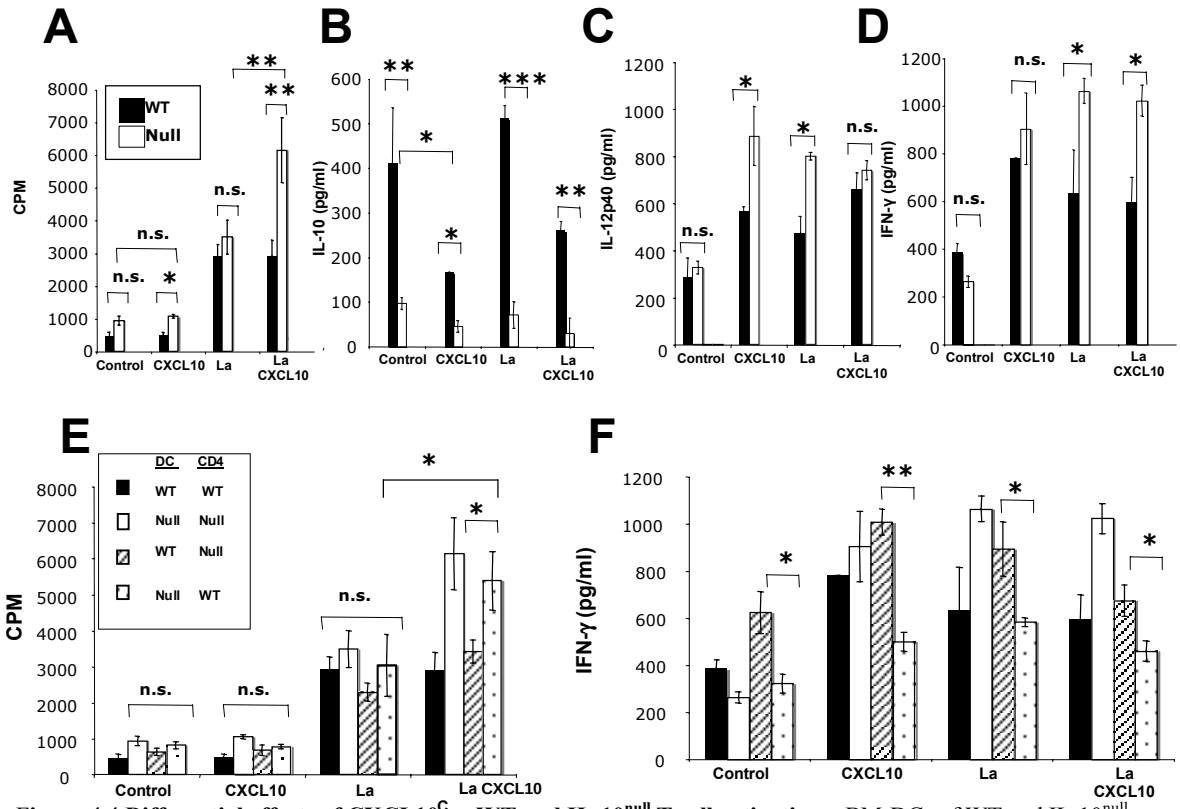


Figure 4.4 Differential effects of CXCL10 on WT and IL-10^{null} T cell activation. BM-DCs of WT and IL-10^{null} mice were infected with *L. amazonensis* (La) promastigotes for 24 h in the absence or presence of CXCL10 (200 ng/ml), treated with mitomycin C (50 mg/ml), and then co-cultured with spleen-derived naïve CD4$^+$ T cells (2×10^6/ml) at a 1:10 DC-to-T ratio. **(A)** After 96 h of co-culture, CD4$^+$ T cell proliferation was measured by a [^{3}H]-thymidine incorporation assay. Culture supernatants were collected at 4 days of stimulation for measuring the levels of IL-10 **(B)** IL-12p40 **(C)** and IFN-γ **(D)** by ELISA. **(E)** Proliferation of co-cultured WT and IL-10^{null} DCs and CD4$^+$ T cells was also measured at the indicated settings. **(F)** Culture supernatants were harvested at 4 days of stimulation and measured for IFN-γ by ELISA. Shown are representative data from one of three independent experiments with similar results. * ($p < 0.05$), ** ($p < 0.01$), and *** ($p < 0.001$) indicate statistically significant differences between the indicated groups.

In addition, we observed that IL-10^{null} CD4$^+$ T cells co-incubated with CXCL10-treated DCs produced more IL-12p40 as compared to their WT T cell counterparts (Fig. 4.4C; $p < 0.05$). Likewise, there was a significant elevation in IFN-γ secretion when IL-10^{null} CD4$^+$ T cells were co-cultured with IL-10^{null} DCs that were previously exposed to *L. amazonensis* alone or *L. amazonensis* plus CXCL10 (Fig 4.4D; $p < 0.05$). Together, these results suggest that in the absence of IL-10 from both DCs and T cells, CXCL10 treatment can markedly promote priming of CD4$^+$ T cells to become antigen-specific Th1 cells.

To determine the source of IL-10, we utilized a cross-coculture system in which IL-10 was eliminated from either $CD4^+$ T cells or DCs. As shown in Fig. 4.4E, there was a significant increase in proliferation of WT $CD4^+$ T cells when IL-10 was eliminated from *L. amazonensis*-infected, CXCL10-treated DCs (dotted bars) in comparison to IL-10^{null} $CD4^+$ T cells incubated with *L. amazonensis*-infected, CXCL10-treated WT DC (hatched bars; $p < 0.05$) and *L. amazonensis*-infected, IL-10^{null} DCs ($p < 0.05$), suggesting that IL-10 inhibited DCs to properly signal $CD4^+$ T cells to expand. In addition, we consistently observed that eliminating IL-10 from $CD4^+$ T cells significantly impacted T cell IFN-γ production (Fig. 4.4F; comparing hatched bars with dotted bars in all tested groups $p < 0.05$, $p < 0.01$). Collectively, these data illustrate the important role of IL-10 in modulating both DC and T cell functions.

CXCL10 STIMULATES $CD4^+$ T CELLS TO EXPRESS THE IL-12Rβ_2 CHAIN AND PRODUCE IFN-GAMMA.

L. amazonensis-infected mice are deficient in Th1 cell activation (126, 127) due to an IL-4-independent downregulation of the IL-12Rβ_2 chain on $CD4^+$ T cells (125, 126). We therefore examined whether CXCL10 treatment could overcome these deficiencies on $CD4^+$ T cells. $CD4^+$ T cells were isolated from the spleen of naïve mice, treated with 200 ng/ml of CXCL10 for 24 h, and subjected to RT-PCR analysis. While IL-12Rβ_2 mRNA was undetectable in untreated naïve $CD4^+$ T cells (Fig. 4.5A, lane 1), CXCL10 treatment resulted in a 2.5-fold increase in the IL-12Rβ_2 chain expression (Fig. 4.5A & B, lane 3).

To determine if CXCL10 could increase the expression of the IL-12Rβ_2 chain on *in vivo* primed $CD4^+$ T cells, we isolated $CD4^+$ T cells from DLN of B6 mice that were infected with *L. amazonensis* for 6 to 8 wk. These cells were either cultured in medium

for 24 h or treated with CXCL10 for 24 h in the absence of APCs and antigen prior to RNA isolation.

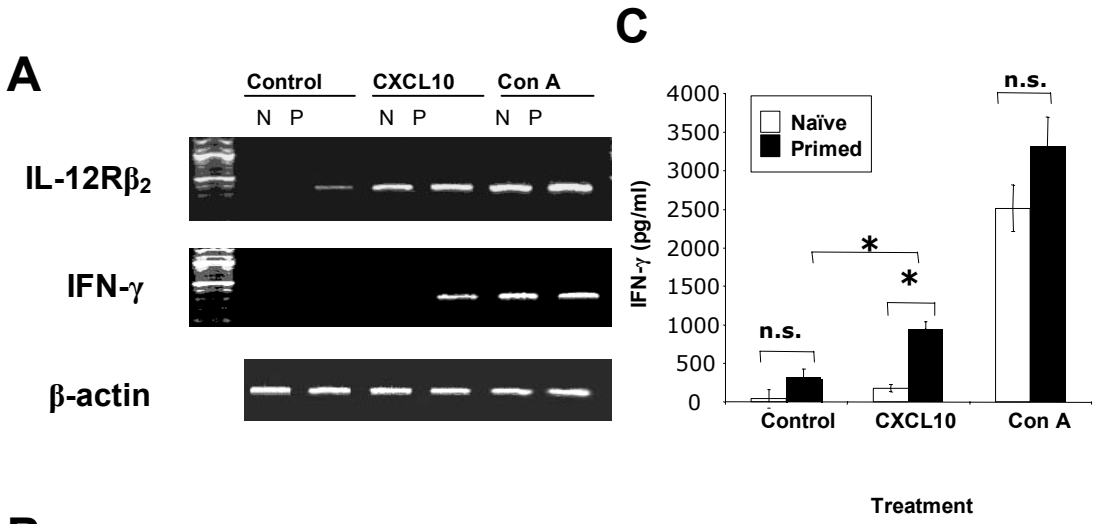


Figure 4.5 CXCL10 directly stimulates CD4$^+$ T cells to express the IL-12Rβ$_2$ chain and produce IFN-γ. Naïve CD4$^+$ T cells (N) and *L. amazonensis*-primed CD4$^+$ T cells (P) were isolated from the spleen of naïve or DLN of L amazonensis-infected B6 mice (8~10 weeks), respectively. CD4$^+$ T cells were cultured in the presence or absence of CXCL10 (200 ng/ml) or Con A (2 ng/ml) for 24 h. (A) Total RNA (100 ng) was extracted for RT-PCR analyses for IL-12β$_2$, IFN-γ, and β-actin. (B) Bands of interest were analyzed via spot densitometry and normalized with their corresponding β-actin controls. Data are presented as fold of increase in CXCL10-treated groups over medium controls, and shown is representative of three independent experiments. (C) The levels of IFN-γ in culture supernatants were assayed by ELISA. Results are shown as mean ± SD from a total of three independent experiments performed in duplicate. * ($p < 0.05$) represents statistically significant differences between the indicated groups, and n.s. indicates groups that were not statistically significant ($p > 0.05$).

Although primed CD4$^+$ T cells expressed low levels of IL-12Rβ$_2$ but not IFN-γ (lane 2), CXCL10 treatment led to 3.5- and 2-fold increase in IL-12Rβ$_2$ and IFN-γ expression (lane 4), respectively. These data suggest that CXCL10 can directly stimulate the expression of the IL-12Rβ$_2$ chain, making CD4$^+$ T cells more responsive to IL-12. Although CXCL10 is a downstream molecule induced by IFN-γ, CXCL10 can, in turn,

trigger strong IFN-γ gene expression via antigen-dependent and –independent means (203). Consistent with this notion, we found that *in vivo* primed, CXCL10-treated $CD4^+$ T cells produced significant amount of IFN-γ (Fig. 4.5C; $p < 0.05$). These data suggest that CXCL10 can enhance T cell responsiveness to IL-12 and stimulate IFN-γ production.

DISCUSSION

Chronic, non-healing *L. amazonensis* infection in mice have been linked to both impaired production of IL-12 and unresponsiveness of $CD4^+$ T cells to IL-12 activation, resulting in a diminished Th1 response (125, 129). It has been proposed that *L. amazonensis* parasites can evade host immunity by inhibiting these early responses (129), thus preventing the expansion of antigen-specific Th1 cells even in the absence of Th2 dominance (204). In this study, we investigated the effects of CXCL10 treatment on both DCs and $CD4^+$ T cells and their potential influence up *L. amazonensis* infection in mice. While much has been learned regarding the role of CXCL10 in T cell chemotaxis (203), this study is focused mainly on the ability of CXCL10 to function in cellular activation of both DCs and $CD4^+$ T cells in *L. amazonensis* infection. Using an *in vitro* DC infection and T cell priming system, we have provided evidence that CXCL10 treatment can trigger significant production of IL-12p40 but reduction of IL-10 in DCs, independent of parasite infection (Figs. 4.1 and 4.2). In the presence of exogenous CXCL10, infection with *L. amazonensis* promastigotes preferentially stimulates DCs to produce significant higher levels of IL-10 in comparison to cells treated with CXCL10 or parasite alone (Fig. 4.3). The role of endogenous IL-10 in DC and T cell activation during *L. amazonensis* infection and the interplay between IL-10 and CXCL10 was further investigated using DC and $CD4^+$ T cells that were derived from $IL\text{-}10^{null}$ mice (Figs. 4.3 and 4.4). This study

provides the first evidence that while eliminating endogenous IL-10 from DCs greatly enhance DC IL-12 production and its APC functions, eliminating IL-10 from both DCs and $CD4^+$ T cells allow maximal T cell proliferation and IFN-γ production during *L. amazonensis* infection (Fig. 4.4). Collectively, this study indicates that *L. amazonensis* parasites have certain intrinsic features that favor the production of IL-10 over IL-12 in DCs, even in the presence of exogenous, Th1-promoting CXCL10. In addition, this study suggests that the direct effect of CXCL10 on $CD4^+$ T cells in stimulating the expression of the IL-12Rβ_2 chain and IFN-γ production may collectively contribute to the protective effect of CXCL10 treatment *in vivo* (197).

IL-10 is known to inhibit the induction and expression of cell-mediated immunity required for the control of many intracellular pathogens, including *L. major*, *Toxoplasma gondii,* and *Trypanosoma cruzi* (121, 205, 206). IL-10 can be produced by many cell types, including MΦs, conventional DCs, T cells, B cells and NK cells (207). At the DC level, IL-10 can act against Th1 development by blocking IL-12 production, interfere with DC maturation, and modulate apoptosis (208). It has been shown that IL-10^{null} B6 WT mice or B6 mice treated with anti-IL-10 receptor Abs achieved sterile cure to *L. major* infection (209). We and others have reported that susceptibility to *L. amazonensis* was not solely dependent on the expression of IL-10, since these parasites persisted in IL-10^{null} mice, despite an enhanced Th1 response at early and late stages of infection (126, 127). While IL-10 production in *Leishmania* infection is crucial for MΦ activation and disease exacerbation (210), we observed that IL-10^{null} DCs have an enhanced capacity to produce IL-12 (Fig. 4.3), which was greatly amplified in *L. amazonensis*-infected, CXCL10-treated IL-10^{null} DCs (Fig. 4.3B). These data suggest that in WT DCs, despite the beneficial effects afforded by CXCL10 treatment, exogenous CXCL10 can only

partially overcome IL-10-mediated suppressive effects in *L. amazonensis*-infected DCs (Fig. 4.2). Our results are consistent with a report that described augmented inflammation by MΦs with a specific deletion of the IL-10 exposed to LPS (211). These results show the direct influence of IL-10 upon DC function throughout the duration of an immune response to pathogens.

In the present study, we also examined the importance of DC- versus T-cell-derived IL-10 in the host response to *L. amazonensis* infection. We have shown that the magnitude of antigen-specific T cell proliferation and T cell cytokine production (IL-12p40 and IFN-γ) reached their highest levels when endogenous IL-10 was eliminated from both DC and $CD4^+$ T cells (Fig. 4.4, open bars). These observations are consistent with a previous report (191), showing that in the absence of IL-10, CXCL10 treatment impacts T cell expansion, which is often defective in *L. amazonensis*-infected mice. Interestingly, we also found that while eliminating IL-10 from DCs greatly enhanced DC IL-12 production from all three treatment groups (CXCL10, *L. amazonensis* infection, and CXCL10 plus infection, Fig. 4.3), limiting IL-10 from the T cell source had more profound effects on T cell IFN-γ production in all these three treatment groups (Fig. 4.4, hatched bars versus dotted bars). These observations are consistent with a previous report describing the IL-10-deleted T cells secreted substantial amounts of proinflammatory cytokines after *in vitro* activation with *Toxoplasma gondii* (212). Together, our *in vitro* studies presented herein support our previous studies (127), indicating that during natural *L. amazonensis* infection in an immunocompetent host, endogenous IL-10 from both DCs and T cells contribute to impaired cell-mediated immune responses to this parasite. Since $IL\text{-}10^{null}$ mice remain susceptible to *L. amazonensis* infection, developing persistent infection (68, 73), it is possible that IL-10 is not the sole factor, and that other

suppressive, regulatory cytokines such as TGF-β (62) or other undefined mechanisms may also contribute to host susceptibility to this parasite.

CXCL10 is a CXC chemokine known to favor the recruitment and activation of Th1-polarized cells (180). IFN-γ-producing Th1 cells most commonly express CXCR3 and CCR5, while IL-4/IL-5-producing Th2 cells are more often characterized by expression of CCR4 (148). CXCL9, CXCL10 and CXCL11 are all secreted by endothelium, epithelium, fibroblasts, keratinocytes, neutrophils, monocytes, and DCs and are chemotactic for leukocytes, especially activated T cells (154). Currently, there are several studies on the effect of CXCL10 in the control of infections caused by intracellular pathogens and those concerning *Leishmania* infections have reached contradicting conclusions. For example, an early and strong induction of the CXCL10 gene strongly correlated with the healing phenotype in *L. major*-resistant B6 mice (171, 213); however, a single injection of CXCL10 into *L. major*-susceptible BALB/c mice resulted in enhanced lesion development (169). Furthermore, *L. major*-infected $CXCR3^{null}$ mice were capable of mounting an efficient Th1 response since increases in Th1-associated IgG2a and significant IFN-γ and IL-12 production by the DLN cells restricted systemic spread of infection, yet failed to control parasite replication at the site of infection and develop chronic non-healing lesion (180). Indeed, *L. major* releases an inhibitory factor, which interferes with the release of CXCL10 in effort to prevent NK cell activation (172). Therefore, most studies suggest a beneficial role of CXCL10 in cutaneous leishmaniasis caused by *L. major* infection (169, 180, 213) and visceral leishmaniasis caused by *L. donovani* (214).

Non-healing cutaneous leishmaniasis caused by *L. amazonensis* infection in mice

is marked by an IL-4-independent down-regulation of the IL-12Rβ_2 chain on $CD4^+$ T cells and lack of responsiveness to IL-12 treatment (126, 129). It is possible that they may be due to suppressed NO in *L. amazonensis*-infected hosts, a well-known inducer of the IL-12Rβ_2 chain (215). We found that CXCL10, when given to purified naïve or *L. amazonensis*-primed $CD4^+$ T cells, markedly enhanced mRNA transcripts of the IL-12Rβ_2 chain (Fig. 4.5). It has been postulated that CXCL10 may be relevant in human immune responses, not only for its role in the chemotaxis of T cells to inflammatory sites, but also by regulating the expression of cytokine synthesis patterns. Furthermore, CXC10 binding to its receptor was found to act as a co-stimulus with antigen, resulting in substantially enhanced recall IFN-γ production, which inhibited T cell proliferation (216). In the present study, we also observed a significant increase in IFN-γ production in *L. amazonensis*-primed, CXCL10-treated $CD4^+$ T cells (Fig. 4.5C). Although subcutaneous injection of CXCL10 did not completely resolve *L. amazonensis* infection in susceptible mice (197), our current findings suggest that by eliminating IL-10, CXCL10 treatment of mice infected with *L. amazonensis* could potentially render these mice more resistant. *In vivo* infection and CXCL10 treatment studies using IL-10^{null} mice are ongoing in the lab.

In summary, we report in this study that CXCL10 treatment could directly increase IL-12p40 production, as well as APC function in DCs. *L. amazonensis*-infected DCs also secreted significant amounts of IL-10, which limited the efficacy of CXCL10-induced IL-12p40 production. The elimination of IL-10 led to the enhanced production of IL-12 in DCs and $CD4^+$ T cell proliferation and IFN-γ production in groups treated with CXCL10. In addition, exogenous CXCL10 led to increased expression of the IL-12Rβ_2 chain on $CD4^+$ T cells, resulting in enhanced IL-12 responsiveness and subsequent IFN-γ production. Treatments that specifically target *L. amazonensis*–mediated

downregulation of the IL-12 receptor, leading to an increase in IFN-γ production could result in the development of an effective therapy against the formation of disease syndromes.

CHAPTER 5: SUMMARY AND DISCUSSION

A majority of experimental data gathered in this dissertation has been presented as independent manuscripts in Chapters 3 and 4. Conclusions specific to those chapters were summarized and thoroughly discussed within both. In this final chapter, we would like to discuss some unpublished data and to expand upon those conclusions in a broader context of *Leishmania* infection. Additionally, we will indicate and consider the pitfalls in some of our experiments, as well as elaborate on potential directions that might be undertaken as a result of this dissertation. Further discussion of these findings and their implications for the future *Leishmania* research are described below.

MACROPHAGES FROM DIFFERENT MURINE BACKGROUNDS AND THEIR T-INDEPENDENT RESPONSES

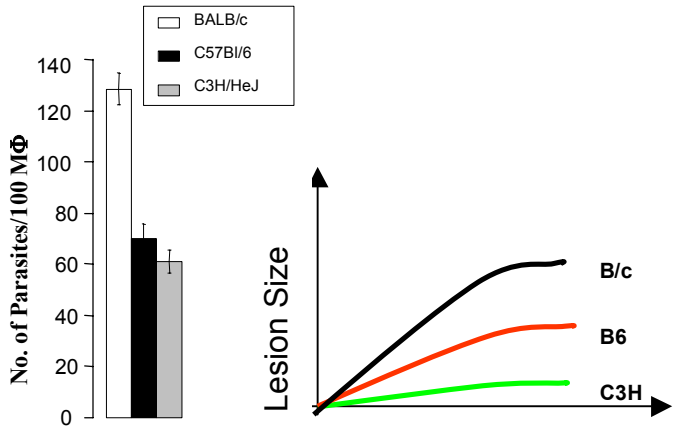


Figure 5.1 Macrophages from different backgrounds have altered parasite loads. (A) BM-MΦs of BALB/c, C57Bl/6 or C3H/HeJ mice were seeded in 24-well plates (3×10^5 cells/coverslip/well). Cells were infected with 2.4×10^6 stationary-phase promastigotes (at 8:1 parasite-to-cell ratio). All groups of cells were subsequently stained for parasites, using pooled sera from infected mice and FITC-conjugated, goat-anti mouse IgG. The nuclei of the cells were stained with DAPI. Images from 10 random fields per condition were taken for counting parasite loads. Data are presented as number of parasites per 100 MΦs. (B) Graphic representation of lesion development due to *L. amazonensis* infection in BALB/c, C57Bl/6 or C3H/HeJ mice.

As previously discussed in Chapter 3, we observed a significant decline in parasite burden in CXCL10-treated MΦ, irrespective of mouse background utilized. This

finding was intriguing given that this series of experiments were performed in the absence of T cells, suggesting that MΦs generated from different backgrounds may possess intrinsic responses against the parasite which are unique. For example, Oliveria et al. (2005) reported that *L. major*-infected MΦs derived from C3H mice produced significantly elevated IL-12 compared to both BALB/c and C57BL/6 with high IL-12p40 in BALB/c linked with increased susceptibility. In addition, Carrerra et al. (1996) reported that LPS stimulated C3H MΦs produced increased TNF-α and IL-1α/β compared to BALB/c mice, further implying that MΦs generated from this background are more apt towards pro-inflammation compared to their BALB/c counterparts. In terms of *L. amazonensis* infections, this is an important point to consider given that $CD4^+$ T cells are considered pathogenic and therefore, therapies specifically target MΦ effector functions could be key in developing an effective treatment.

PARASITE KILLING VERSUS INHIBITION OF REPLICATION

As discussed in Chapter 3, BM-MΦs express CXCR3 and engagement with its ligand CXCL10 triggers the production of pro-inflammatory mediators, such as NO, IL-12, CCL12, CCL3, and CXCL2 (Fig. 3.4). This enhanced MΦ activation resulted in the decline of parasite numbers within CXCL10-treated MΦs that were infected with either promastigotes (Fig. 3.2) or amastigotes (Fig. 3.3). However, it remained unclear whether this reduction was due to CXCL10-mediated parasite killing or to the inhibition of parasite replication. We observed a significant reduction in parasite burden between MΦs treated with CXCL10 and infected with *L. amazonensis* for 48 h versus 5 h. Given the reduced parasite loads between two time points, this data indicate that CXCL10 treatment of MΦs initiated parasite killing, partially due to NO production (Fig. 3.4B), but as a whole, the precise mechanism remains undetermined. However, caution must be

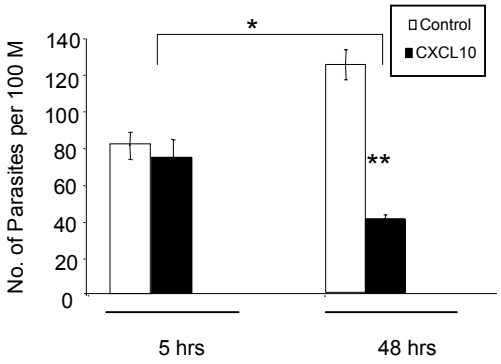
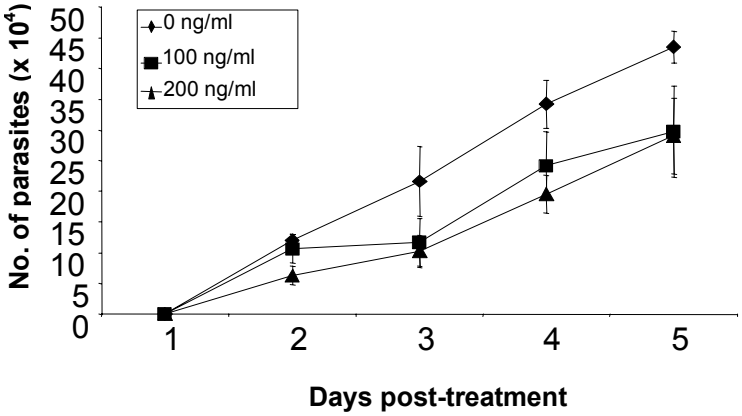


Figure 5.2 CXCL10-treatment induces parasite killing in MΦ. BM-MΦs of BALB/c mice were seeded in 24-well plates (3×10^5 cells/coverslip/well). Cells were untreated or treated with CXCL10 (100 ng/ml) for 4 h prior to infection with 2.4×10^6 stationary-phase promastigotes. At 5 or 48 h post-infection, cells were subsequently stained for parasites, using pooled sera from infected mice and FITC-conjugated, goat-anti mouse IgG. The nuclei of the cells were stained with DAPI. Images from 10 random fields per condition were taken for counting parasite loads. Data are presented as number of parasites per 100 MΦs, and shown is mean ± SD of three independent experiments (representing 30 fields per condition). (* $p < 0.05$; ** $p < 0.01$).

applied to this conclusion since only two time points were utilized, and we were unable to rule out the capacity of CXCL10 to inhibit parasite replication once MΦs were infected. Another possibility is that CXCL10 may be toxic to *L. amazonensis* and can mediate its effects independent of the host immune response. This speculation was based upon our experiments in which we treated promastigotes in cell free media with 100 or 200 ng/ml of CXCL10 (Fig 5.2)



F**igure 5.3 CXCL10-treatment reduces parasite growth in cell-free medium.** CXCL10 (100 and 200 ng) was given to 1 x 10^4 L. amazonensis promastigotes cultured in a 48 well plate in triplicate. Each well was counted daily and the displayed data is representative of the average of the three wells. $p > 0.05$.

Roychoudhury et al. (2006) reported the existence of a chemokine-binding property amongst *Leishmania* and *Trypanosoma* parasites, indicating the presence of a chemokine-binding molecule on the parasite surface. Although they hypothesized that the presence of such a marker would provide the parasite with a survival advantage, it is

also possible that engagement with chemokines could be potentially detrimental. In CXCL10-treated promastigotes, we observed numerous 'non-healthy' and dead parasites (Fig. 5.2). Over a 5 day period, an observable reduction in the number of parasites compared to untreated control was readily seen; however, statistical significance was not achieved. Therefore, we felt that this series of experiments did not conclusively establish whether CXCL10 was indeed toxic to the parasites. The data could also be interpreted to suggest that application of the chemokine retarded parasite replication instead of resulting in their demise. One approach to address this question is via flow cytometric analysis of CFSE labeled parasites to measure the number of parasite generations that occur after CXCL10 treatment. This experiment should reveal any measurable changes in parasite replication due to CXCL10 treatment, thereby clarifying its role in causing direct versus indirect reduction of parasite numbers. Understanding this mechanism could better describe the contributions of CXCL10 in *L. amazonensis* infections and in other diseases caused by intracellular microorganisms as well.

DNA-BASED VERSUS RECOMBINANT PROTEIN-BASED TREATMENT

Throughout the course of this dissertation study, we used recombinant CXCL10 in all *in vitro* and *in vivo* experiments. As previously discussed in Chapter 3, the half-life of recombinant CXCL10 *in vivo* may not be sufficient to provide long-lasting protection. The use of a CXCL10/Ig fusion protein or CXCL10-expressing plasmid may partially overcome this problem. Since CXCL10 treatment triggered increases in both DC production of IL-12p40, as well as $CD4^+$ T cell responsiveness to IL-12 (Fig. 4.1 and Fig 4.5), we utilized commercially available CXCL10-expressing plasmid (Invivogen, San Diego, CA) and IL-12-expressing plasmids (generously provided by Hua Yu, Moffitt

Cancer Center, Tampa, FL.) to test the efficacy of these plasmids as a potential immunotherapy in *L. amazonensis*-infected mice. In contrast to our previous observations made with recombinant CXCL10 (See Fig. 3.5), we did not observe a significant decrease in lesion development in mice that received the dual CXCL10/IL-12 DNA plasmid treatment.

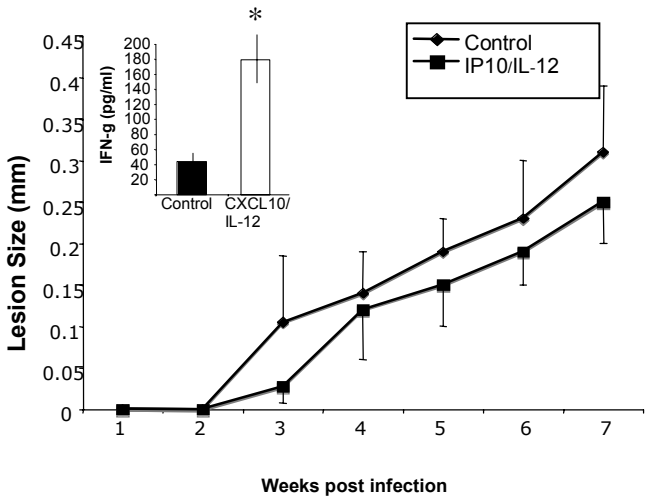


Figure 5.4 Lack of protection of BALB/c mice treated with CXCL10/IL-12 plasmids against *L. amazonensis*. Mice (4/group) were left untreated or treated with the indicated DNA constructs (100 μg) three times at 3- week intervals after challenge with 2 x 10^6 promastigotes. Lesion development was monitored by measurement of footpad thickness, and is expressed as the mean difference between the infected and uninfected footpad for each group. At 7 wk post-infection, DLN cells were cultured and measured for IFN-γ via ELISA. Each group was performed in duplicate. * $p < 0.05$

Several possibilities may explain the lack of success with this treatment. First, given that this experiment was only performed once, optimization is still required to determine the prime therapeutic dose and timing needed to induce protection. Second, it is possible that this treatment strategy may be employed to prime the naïve mice immune response prior to infection instead of attempting to invoke Th1-biased immunity after infection has already been established. Indeed, our lab previously found that introduction of the *Leishmania* nuclease gene P4 and the IL-12 gene provided potent immune protection against challenge with *L. amazonensis* (195). Lastly, although we have presented considerable evidence in support of the protective role of CXCL10 in *L. amazonensis* infections, it is possible that administration of this pro-inflammatory chemokine is insufficient to overcome the parasite-mediated interference with the host's ability to mediate a protective response. Defining the role of known anti-

inflammatory/pathogenic mediators is crucial to understanding the mechanisms employed by the parasite to continue its maintenance within the host.

CXCL10 AND IL-10 REGULATION IN MURINE *L. AMAZONENSIS* INFECTION

As discussed in detail in Chapter 3, treatment of *L. amazonensis* susceptible C57BL/6 mice with CXCL10 resulted in delayed lesion development and diminished parasite burden (Fig. 3.5). This treatment also triggered elevated IL-12 and IFN-γ, both of which are required to mediate the host immune response against intracellular pathogens, specifically *Leishmania* (Fig. 3.6). In determining the potential mechanism of action of CXCL10 treatment (Chapter 4), we found *in vitro* that both DCs and $CD4^+$ T cells respond to CXCL10 by producing increased levels of IL-12p40, as well as becoming more IL-12 sensitive, respectively (Figs. 4.1 and 4.5). Also, we observed a marked increase in IL-10, following *L. amazonensis* infection and CXCL10 treatment, suggesting that IL-10 may be responsible for disease progression in *L. amazonensis*-infected mice.

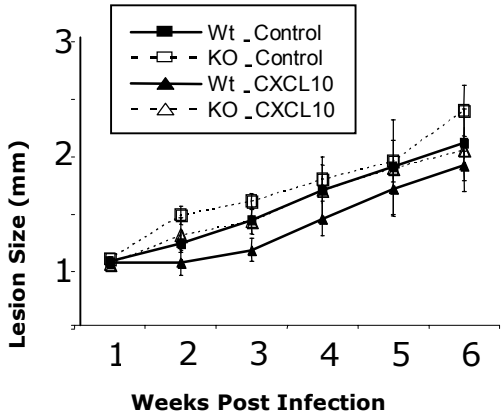


Figure 5.5 Local administration of CXCL10 does not delay lesion development in IL-10^{null} mice. IL-10^{null} mice (4 per group) were infected s.c. with 2×10^6 L. amazonensis promastigotes and treated s.c. with CXCL10 (100 ng in 5 μl) or PBS on days 1, 3 and 7 of infection. Lesion size (in mm) was monitored weekly, and shown is the mean ± SD for each group Results are representative of two independent experiments

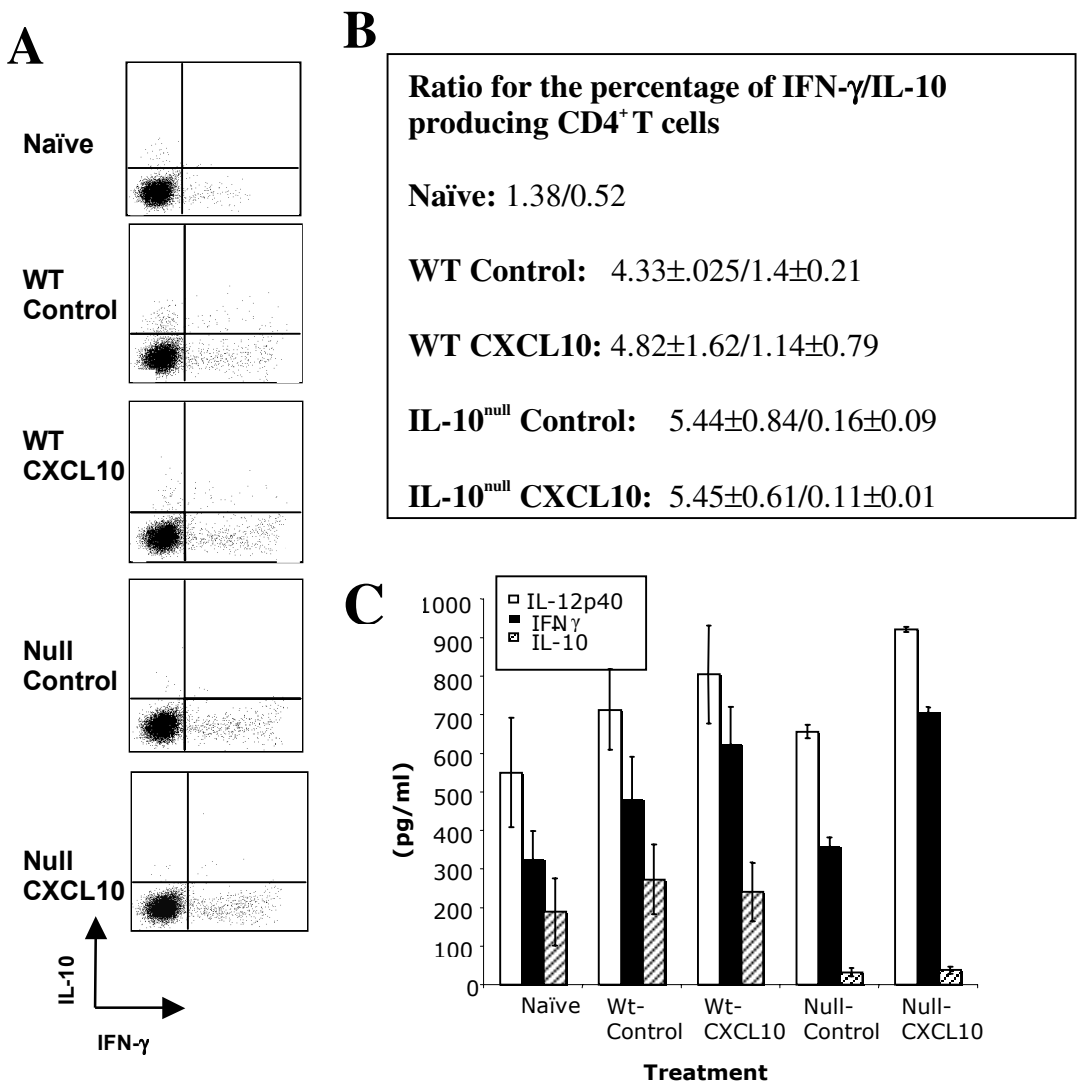


Figure 5.6 CXCL10 did not significantly alter cytokine production in either WT or IL-10^{null} mice. DLN cells from (A) WT and IL-10^{null} B6 mice (3/group) were collected and stained for surface expression of CD4 plus intracellular IFN-γ and IL-10. Shown are percentages of positively stained cells gated on CD4$^+$ cells. Shown are representative data from one experiment. (B) The percentages of IFN-γ and IL-10 are shown as mean ± SD from one independent experiment performed in triplicate. (C) The levels of IL-12p40, IFN-γ and IL-10 in culture supernatants were assayed by ELISA. Results are shown as mean ± SD from a total of three independent experiments performed in duplicate.

Based upon our *in vitro* data, we speculated that after elimination of endogenous IL-10 from susceptible mice, the effect of CXCL10 treatment would be greatly enhanced, potentially resulting in a healing phenotype as seen in *L. major* models. To test this hypothesis/ we infected both WT and IL-10^{null} mice with 2 x 10^6 bulk-cultured *L. amazonensis* promastigotes which were subsequently treated three times (at days 1, 3, and 7 post-infection) with recombinant CXCL10. To our surprise, we observed that

lesion development in CXCL10-treated IL-10^{null} mice was not significantly different compared to either WT or IL-10^{null} control. CXCL10-treated WT mice showed slower lesion development compared to the other groups; however, this difference was also not statistically significant. Limiting dilution analysis revealed that there was no significant change in parasite burden amongst each group.

To understand the reason why did not observe any change between each group, FACS analysis of $CD4^+$ T cells from both CXCL10-treated and untreated WT and KO mice were performed. Slight difference in the percentage of IFN-γ and IL-10-producing cells was seen (Fig 5.6), which was in contrast to our data obtained in Figs 3.5 and 3.6 where, not only was lesion development delayed, but also there was a strong induction of IFN-γ. Furthermore, ELISA analysis did show elevations in both IL-12p40 and IFN-γ in CXCL10 treated groups, suggesting that some effect did occur; however, it was insufficient to provide any significant benefit to those groups of mice. The failure of the present experiment may be due to three possibilities. First, unlike our previous finding, we infected WT and IL-10^{null} mice with 2 x 10^6 bulk stationary-phase promastigotes rather than 2 x 10^5 metacyclic promastigotes. As discussed in detail in Chapter 1, *Leishmania* transforms from poorly infective procyclic promastigotes into highly infective metacyclic promastigotes within the sandfly vector, increasing the parasite's ability to infect its host (10). Metacyclic promastigotes can be experimentally isolated via negative selection utilizing the 3A1 mAb specific against the major *L. amazonensis* cell surface component LPG (173). The advantage of utilizing metacyclic promastigotes is their increased ability to infect and survive within host MΦs, as well as decreased amount of 'sub-optimal' parasites that may be dead or dying at the time of

murine inoculation. Also, inoculation of bulk parasite may result in non-specific inflammation, whereas metacyclic promastigotes trigger infection-mediation inflammation. Due to unforeseen circumstances, the 3A1 mAb was no longer available in our lab, and therefore, we used bulk promastigotes in this mouse study. Second, since we administered a higher dose of parasites (1 log more) in comparison to our previous experiment (Fig. 3.5), it is conceivable that the previous dose of administered recombinant CXCL10 (100 ng given 3 times at 1, 3 and 7 days post-infection) might not be sufficient to garner an appropriate immune response (Figs 3.5 and 3.6). Future attempts at studying the effect of CXCL10 within IL-10^{null} should include a CXCL10 dose curve to determine the concentration needed to achieve maximal effectiveness against *L. amazonensis in vivo*. Lastly, despite our concerns with parasite condition and CXCL10 therapeutic dose, it is conceivable that total elimination of IL-10 from mice could potentially exacerbate disease symptoms caused by *L. amazonensis* due to other mechanisms. As discussed in Chapter 2, our lab has demonstrated that $CD4^+$ $CD25^+$ $Foxp3^+$ T cells are the primary source of IL-10 and that these regulatory T cells can restrain the activation of pathogenic Th1-like lymphocytes (183). It is possible that total removal of endogenous IL-10 resulted in the 'loss of control' over pathogenic $CD4^+$ T cells by regulatory T cells, which allowed *L. amazonensis* infection to continue unchecked despite treatment with CXCL10. In Fig. 4.4, we observed a significant upregulation of IFN-γ when IL-10 was removed from both DCs and $CD4^+$ T cells, as well as $CD4^+$ T cells cultured with WT DCs. This unregulated production of IFN-γ could result in immune-mediated pathologies, which may explain the rise in lesion development

observed in Figure 5.3, as well as explain the failure of CXCL10 treatment to control *L. amazonensis* infection in IL-10^{null} mice.

An alternative approach to test the precise role of IL-10 and its affect upon CXCL10 treatment during murine *L. amazonensis* infections would be to adoptively transfer WT $CD4^+$ $CD25^+$ regulatory T cells into IL-10^{null} mice prior to *L. amazonensis* infection and CXCL10 treatment. Since IL-10 has been shown to favor parasite replication in MΦs (68), as well as interfere with DC APC functions (43), an alternate method could use a conditional cell-type specific knockout of IL-10 in APCs; meanwhile, T cells, including regulatory T cells would maintain their full IL-10 producing capacity. This would provide an opportunity for regulatory T cells to exert control over pathogenic T cells, and at the same time, would give tremendous insight into, not only the mechanism of action of CXCL10 treatment, but also illustrate the contribution IL-10 provides in the scope of *L. amazonensis* infections. Although regulatory T cells were not the focus of this dissertation project, it is apparent that this cell type must be considered when attempting to answer questions concerning immune regulation during *L. amazonensis* infections.

CONCLUDING REMARKS

Although our pilot studies investigating the efficacy of CXCL10 treatment in IL-10^{null} mice were not successful, throughout the course of this dissertation research, we did observe significant decreases in *L. amazonensis* infections both *in vitro* and *in vivo*. First, CXCL10 treatment of MΦs led to decreased parasite burden while increasing the key

effector NO, as well as several other pro-inflammatory mediators. Second, not only did DCs treated with CXCL10 experience a substantial increase in IL-12p40 production, but also this treatment significantly decreased IL-10 secretion. In addition, CXCL10 triggered the upregulation of the IL-12Rβ_2 chain on $CD4^+$ T cells, making them more responsive to IL-12-mediated signaling. Lastly, *L. amazonensis* susceptible C57BL/6 mice showed significant delays in lesion formation and overall parasite burden when locally administered CXCL10. Collectively, data obtained in this dissertation support the beneficial contributions of CXCL10 against infection and potential mechanisms employed by the parasite to avoid the host immune response. The sum of these studies supports the model that a fine balance occurs, not only between the different cell types at site of infection, but also among the cytokine/chemokine network which aid in shaping the proper immune response.

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Publications

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