Leishmania amazonensis and macrophage interactions: immune factors necessary to kill the parasite

by

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For the Major Program
DEDICATION

To my wife, children, parents
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CHAPTER 1

General Introduction

Introduction

*Leishmania* spp. are protozoan parasites known to infect humans and animals. They are obligate intracellular parasites that primarily infect macrophages in the mammalian host but are less host cell specific in the vector host, the sandfly. Leishmaniasis is spread in different parts of the world (about 88 countries), where millions of people either are infected or at risk of being infected. It is considered the third most important parasitic disease after malaria and trypanosomiasis. Clinical outcome of leishmaniasis is dependent on the strain of *Leishmania* and immunological status of the host. Recently, research interest to study *Leishmania* as a disease increased with higher incidence of this disease in immunocompetent people. In the US, there has been more attention to this disease due to roughly 800 cases of leishmaniasis reported in soldiers serving in endemic areas of Iraq and Afghanistan.

Studying *Leishmania* is beneficial not only in regard to disease prevention and treatment, but also for its role in promoting a basic understanding of the immune system. This has particularly been shown in models of *Leishmania* infection in inbred strains of mice where different strains of mice have different response to infection with the same species of *Leishmania* or where different species of *Leishmania* respond differently to infection in the same strain of mice. In this thesis we utilize a variation of responses in C3H mice to different *Leishmania* species.
C3H mice infected with *Leishmania major* heal 10-12 weeks post-infection while *Leishmania amazonensis* develop a persistent chronic lesion. This difference in infection outcome correlates with differences in the immune response induced in vivo to each *Leishmania* spp. The main cell types of the immune system known to be involved in responding to *Leishmania* infection are CD4$^+$ T cells, dendritic cells (DCs), and macrophages. Cell mediated immunity characterized by induction of Th1 polarized CD4$^+$ T cells sensitized by proper activation from DCs, leads to productive macrophage activation including the release of cytotoxic factors necessary to kill the intracellular parasite.

The work presented in this thesis summarizes our studies aimed at understanding the resistance of *L. amazonensis* amastigotes to macrophage killing in vitro in comparison to *L. major* amastigotes infection. These studies showed not only a resistance of *L. amazonensis* to macrophage killing despite identical macrophage activation methods that induce killing in *L. major*, but also that cytotoxic factors required to induce *L. amazonensis* killing are different from those that regularly kill *L. major* and are more potent.

The objective of the first study was to analyze the reasons for differential killing of *L. amazonensis* and *L. major* in bone marrow derived macrophages (BMM) in vitro using LPS and IFN$\gamma$ as an identical activation method. For this study a working hypothesis was developed: *L. amazonensis* is more resistant to macrophage activation in vitro as compared to *L. major* infection under the same activation methods. As the differential resistance of *L. amazonensis* to immune response in C3H mice in vivo compared to *L. major* was demonstrated, the actual mechanism for this resistance in macrophages is not clear. At the same time, *Leishmania* spp. were shown to evade macrophages activation by different mechanisms.
The objective of the second study was to test the ability of draining lymph node (DLN) cells from healed *L. major* to activate BMM to control *L. amazonensis* infection in vitro and to characterize the cell types involved and the mechanism of activation. We hypothesized that DLN cells from *L. major* infected mice induce activation of BMM infected with *L. amazonensis* to control the infection. A previous study from our laboratory determined that healed *L. major* infected C3H mice could heal a secondary *L. amazonensis* infection in vivo. There are no previous reports for LN cells from infected mice to activate macrophages in vitro.

The objective of the third study was to characterize the role of antibodies and Fcγ receptors in macrophage activation to induce killing of intracellular *L. amazonensis*. Our hypothesis for this study is that *Leishmania* infection of macrophages would manipulate expression of Fcγ receptors therefore causing regulation of macrophages activation. A previous role for Fc receptor was shown in antibody opsonized *Leishmania* macrophage invasion and evasion of the immune system, while in the previous chapter of this thesis we showed an antibody dependent activation of macrophages secreted from DLN of *L. major* infected mice. The actual role of *Leishmania* infection on macrophages expression of Fcγ receptor was yet unknown.
Literature review

1. Leishmania

1.1 History and Epidemiology

Cutaneous lesions caused by *Leishmania* were first described in people of the Old World at least two thousand years ago (189), while illustration of lesions have been found on human faces of decorating pottery created 1500 years ago in Peru and Ecuador (130). *Leishmania* lesions were first described as part of modern medicine by Russell in 1756. Subsequently, Leishman reported the identification of the parasite in India in 1900 (14). Leishmaniasis is known by other names including; sore, Aleppo evil, Delhi boil, Baghdad sore, Rose of Jericho, Chiclero's ulcer, uta, espundia (mucosal form), forest yaws, Dumdum fever (visceral form), kala-azar, and black fever (42).

88 countries are endemic for leishmaniasis of which 72 countries are developing countries. About 90% of the visceral leishmaniasis occurs in five countries: Sudan, India, Bangladesh, Nepal, and Brazil. While in only eight countries 90% of cutaneous leishmaniasis occur: Afghanistan, Algeria, Brazil, Iran, Peru, Saudi Arabia and Syria (256, 9).

The estimated global prevalence of leishmaniasis is 12 million people with an incidence of 1.5-2 million cases yearly (about 1-1.5 million cutaneous cases and 500 thousand visceral leishmaniasis cases) (256). Increasing risk factors like urbanization play a major role in higher incidence rates, as prior to 1950 only one third of the worlds population live in cities, whereas today more than 50% of people are city dwellers. This has brought traditionally rural diseases into urban areas (168). Climatic changes can also modify geographical distribution of vector, as the distribution of *Leishmania* is mainly determined by
the distribution of sandfly vectors (*Phlebotomus* sp and *Lutzomyia* sp), which is specifically defined by particular environmental breeding habitats.

Recently, emergence of HIV*/Leishmania* co-infection reported in 34 countries globally, has vastly increased the risk of *Leishmania*-infected people for developing visceral illness, particularly in Southern Europe (8, 205)

### 1.2 Strains (New World and Old World)

*Leishmania* species are generally considered to be indistinguishable morphologically. Extrinsic characters, such as clinical features of disease, geographical distribution, and behavior in laboratory animals or vectors and intrinsic ones; immunological, biochemical, or molecular criteria are used for identification (56) (Table 1). *Leishmania* has four clinical forms: cutaneous (CL), mucocutaneous (MCL), diffuse cutaneous (DCL), and visceral (VL). The clinical expression of leishmaniasis depends not only on the genotypic potential of the different parasites, but also on the immunological status of the patient as the cutaneous form can transform to the visceral form in immuno-competent patients (6, 144).
Table 1 Taxonomy and classification of *Leishmania* (adapted from [154])

<table>
<thead>
<tr>
<th>Kingdom</th>
<th>Protista</th>
<th>Disease form*</th>
</tr>
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<tbody>
<tr>
<td>Subkingdom</td>
<td>Protozoa</td>
<td></td>
</tr>
<tr>
<td>Phylum</td>
<td>Sarcomastigophora</td>
<td></td>
</tr>
<tr>
<td>Subphylum</td>
<td>Mastigophora</td>
<td></td>
</tr>
<tr>
<td>Class</td>
<td>Zoomastigophora</td>
<td></td>
</tr>
<tr>
<td>Order</td>
<td>Kinetoplastida</td>
<td></td>
</tr>
<tr>
<td>Family</td>
<td>Trypanosomatidae</td>
<td></td>
</tr>
<tr>
<td>Section</td>
<td>Salivaria</td>
<td></td>
</tr>
<tr>
<td>Genus</td>
<td><em>Leishmania</em></td>
<td></td>
</tr>
<tr>
<td>Species</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>L. mexicana</em></td>
<td>CL</td>
</tr>
<tr>
<td></td>
<td><em>L. venezuelensis</em></td>
<td>CL</td>
</tr>
<tr>
<td></td>
<td><em>L. peruviana</em></td>
<td>CL</td>
</tr>
<tr>
<td></td>
<td><em>L. lansoni</em></td>
<td>CL</td>
</tr>
<tr>
<td></td>
<td><em>L. panamensis</em></td>
<td>CL</td>
</tr>
<tr>
<td></td>
<td><em>L. braziliensis</em></td>
<td>CL, MCL</td>
</tr>
<tr>
<td></td>
<td><em>L. amazonensis</em></td>
<td>CL</td>
</tr>
<tr>
<td></td>
<td><em>L. pifanoi</em></td>
<td>CL, DCL</td>
</tr>
<tr>
<td></td>
<td><em>L. chagasi</em></td>
<td>VL, CL</td>
</tr>
<tr>
<td></td>
<td><em>L. infantum</em></td>
<td>VL</td>
</tr>
<tr>
<td></td>
<td><em>L. donovani</em></td>
<td>VL</td>
</tr>
</tbody>
</table>

*CL: Cutaneous, MCL: Mucocutaneous, DCL: Diffuse cutaneous, VL: Visceral.

1.3 Life cycle and transmission

*Leishmania* parasites exist in two morphological forms, amastigotes and promastigotes, which are found in the mammalian host and sandfly, respectively. Amastigotes entering into the sandfly midgut after a bloodmeal initially transform into procyclic promastigotes. Procyclic promastigotes are non-infective and rapidly divide in the sandfly midgut. They then transform into infective metacyclic promastigotes within 4-7 days, which migrate to the pharynx of the sandfly. There are 6 developmental forms of promastigotes distinguished morphologically: procyclic, nectomenad, leptomonad,
haptomonad, paramastigote, and metacyclic promastigotes (82, 203). Following the bite of an infected sandfly, *Leishmania* metacyclic promastigotes undergo phagocytosis by host macrophages or dendritic cells through binding to various receptors including the complement receptors and Fc receptors (discussed in section 2.1) (147). Once a promastigote is inside a phagosome, fusion with lysosomes and endosomes occurs forming a phagolysosome in which promastigotes transform to amastigotes. The multiplication of amastigotes within macrophages causes eventual rupture of the macrophage and allows spread of infection to other macrophages. The *Leishmania* life cycle is completed when a sandfly feeds on blood of an infected host taking up the amastigotes (90).

The endemic transmission cycle of *Leishmania* typically occurs through the sandfly and mammalian reservoirs including rodents and canines however, humans can be incidental hosts. More recently, with HIV infection a new mode of transmission was recorded via needle between HIV infected people (161).

1.4 Vector and sandfly saliva

Sandflies, the vector for *Leishmania* infection, belong to the family of Psychodidae and subfamily Phlebotominae. There are about 700 species of sandflies of which 70 are known to be able to serve as vectors for leishmaniasis (125).

Only the female sandflies transmit the disease as they are hematogenous (feed on blood), they breed and lay eggs in environments where the larvae can find appropriate organic matter, heat and humidity. Immature Phlebotmines are found in organically rich moist soils (*Lutzomyia spp.*.) or contaminated soil of animal shelters (*Phlebotomus spp.*), as
well as in the burrows of certain rodents, in the bark of old trees, in ruined buildings and in cracks of house walls (reviewed in (71)). In searching for a blood meal, usually in the evening and at night, the female sandfly can cover a radius of a few to several hundred meters (107).

Sandfly species are selective to which species of *Leishmania* can develop in their midgut. This specificity is dependent on cellular midgut receptors with specificity to a particular *Leishmania* species lipophosphoglycan (LPG). For example, *L. amazonensis* is transmitted by *Lu. flaviscutellata*, and *L. major* is transmitted by *P. papatasi*, based on their LPG structure (reviewed in (208)).

When sandflies feed on the host blood they probe the skin creating a hemorrhagic pool. It has been shown that the parasite influences sandfly feeding behavior by increasing probing. For example, 11 attempts of a single infected sand fly was recorded (reviewed in (107)). The main purpose of sandfly saliva is to induce vasodilation, prevent blood clotting, and promote immunosuppression. Saliva contains potent vasodilators including maxadilan (136) adenosine (202), and anti-platelet aggregation factors like apyrase (244) and prostaglandin E2 (201). Sandfly saliva components can also play a role in host immunosuppression. Several studies have shown that co-injection of salivary gland homogenates with *Leishmania* infection increase lesion size and exaggerates the infection outcome (16, 68, 141). Saliva has been shown to induce IL-4 production and inhibition of antigen presentation, IFN-γ, iNOS and NO production (89, 123). Maxadilan, which is found in the saliva of *Lu. longipalis* (vector for *L. chagasi*), was found to inhibit T cell proliferation, and prevent delayed type hypersensitivity (DTH) response (195), as well as inhibit TNF-α expression by macrophages and increase IL-6, IL-10 and prostaglandin E2
production (220). Adenosine, which is found in both *P. papatasi* (vector for *L. major*) and *Lu. longipalis* saliva, induced enhancement of IL-10 production and suppression of IFN-γ, TNF-α, IL-12 and NO production (94, 95, 134). The role for *L. amazonensis* vector (*Lu. flaviscutellata*) saliva is unknown yet, as the raising of this sandflies species in the laboratory is difficult.

The role of sandflies saliva in *Leishmania* virulence can be seen when an infection can be established with few number of parasites transferred by the sandfly bite. On the other hand, new vaccines is being developed against components of the sandfly saliva based on the fact that people in areas endemic to *Leishmania* and exposed to multiple bites of sandflies are less likely to be infected with *Leishmania*.

### 1.5 Surface determinants and virulence factors

The expression of the surface components of *Leishmania* are species and stage specific. These components, in part, determine the virulence for the parasite in the vector and mammalian host (reviewed in (173)).

#### 1.5.1 Lipophosphoglycan (LPG)

LPG is a major component of the promastigote surface but is found in low to non-detectable levels in amastigotes (150). It is composed of 4 subunits: 1) oligosaccharide cap, 2) polymer of repeating disaccharide-phosphate units, 3) glycan core and 4) phosphatidylinositol (GPI) anchor. Both the anchor and glycan core are conserved between *Leishmania* species while differences between species occur in the phosphorylated
oligosaccharide repeat branches and in the relative abundance of the cap structures (110).

Structural differences in LPG are also seen in between stages of parasites, for example the length of LPG on *L. major* metacyclic promastigotes is almost twice that of procyclic promastigotes due to an additional side chain modification (152). These changes in LPG affect both the thickness and the type of attachment to the sandfly midgut (191). Conversely, there are no structural changes in *L. mexicana* LPG between stages of the promastigote (112), at the same time a lower level LPG in the promastigotes is seen when they reach the stationary phase (197).

LPG was found to both play a role in resistance to complement and to scavenge oxygen radicals in vitro (226). LPG was also found to inhibit the rate of parasitophorous vacuole maturation (60). LPG-deficient *L. major* parasites were found to be less virulent both in vivo and in vitro (225, 227). In contrast, LPG deficient *L. mexicana* were as virulent as wildtype, which indicates that LPG does not play a role as a virulence factor in *L. mexicana* (108). LPG can activate macrophages through Toll-like receptor-2 and promote synthesis of IFN-γ and TNF-α (55) and to stimulate iNOS expression (193). Studies have suggested the down regulation of LPG expression in the amastigotes may play a role in evading macrophage activation. Finally, minimal role for LPG as virulence factor in *L. mexicana* complex compared to *L. major* parasites was reported.

1.5.2 Proteophosphoglycans (PPG)

PPG is a large family of extensively glycosylated proteins (162). There are three forms of PPG found in *Leishmania*; secreted filamentous PPG (fPPG) and a putative GPI-anchored cell-associated form (mPPG) both in promastigotes, while amastigotes have one
secreted form (aPPG) (111). fPPG is a highly viscous mesh within which the parasite is embedded. It is believed that the gel-like matrix formed by fPPG trap the promastigotes inside the sandflies anterior gut. *L. mexicana* amastigotes secret more aPPG than *L. major*, this secretion inside parasitophorous vacuole cause the increase of vacuole size (188), and for that reason *L. mexicana* complex have large vacuoles.

PPG-null mutant *L. major* parasites are less virulent and are more sensitive to complement lysis. Mice infected with PPG null mutant parasites do not develop lesions. PPG plays a role in scavenging oxygen radicals (227). In contrast, the PPG mutant *L. mexicana* is as virulent as wildtype parasites in both in vitro macrophage assays and in murine infection models (109).

1.5.3 Leishmanolysin (Gp63)

Gp63 is a zinc metalloproteinase that is highly expressed on promastigotes, particularly stationary phase parasites. This stage-specific expression of different isotypes is regulated by changes in mRNA stability and turnover (reviewed by (259)).

Gp63 has been shown to hydrolyze complement components which attach to the parasite surface for opsonization by C3 degradation and converting C3b to inactive C3bi (22). Also, gp63 acts as a complement ligand for the parasite to enter the macrophages via complement receptor 3 (21). Gp63-deficient *L. major* develops normally in sandflies but they are more sensitive to complement. There is a delay in lesion formation in BALB/c mice after infection with these mutant parasites (121).

Mutant *L. amazonensis* which have been genetically engineered to over-express gp63 showed an enhanced capacity to migrate through extracellular matrix in vitro by degrading
collagen IV and fibronectin (153). In contrast, *L. amazonensis* gp63 null mutants have lower macrophage binding capacity and decreased intracellular growth (41). It seems that gp63 plays a role in the intracellular survivability of *L. amazonensis* but not *L. major*.

1.5.4 Glycosylinositolphospholipids (GIPL)

GIPL are free glycosylphospholipids present in all stages of *Leishmania*. They are produced by all trypanosomatids suggesting their function as housekeeping molecules proposed to be important in maintaining plasma membrane (151). GIPL have been shown to be important for parasite invasion in macrophages by binding with serum mannose-binding protein or macrophage mannose-fucose receptor (235). Purified GIPL was found to modulate signal transduction pathway in macrophages by inhibiting PKC and NFκB/c-rel (235), which results in inhibiting IL-12 production (190) and iNOS production (193, 234).

Mutation of *L. amazonensis* GIPL did not affect growth of extracellular promastigote stage of the parasite; instead reduced infectivity towards macrophages and decreased intracellular growth of axenic amastigotes were found (79). At the same time, mutant *L. mexicana* GIPL promastigotes grew normally in culture, but they were also less virulent than wildtype parasites in macrophages and susceptible mice (113). In contrast, the virulence of both amastigotes and promastigotes of *L. major* GIPL mutants was not affected (262).

1.5.5 Cysteine peptidases (CP)

Cysteine peptidases, also called cysteine protease, are group of enzymes produced in both stages of *Leishmania* and stored in the megasome, a large lysosome, of amastigotes or the multi-vesicular tubule-lysosome in promastigotes. The majority of studies preformed on
CP function were done on three forms of the enzymes; CPA, CPB, and CPC, all of which were identified as virulent factors for L. mexicana (reviewed in (169)). L. amazonensis and L. mexicana CPs were described to be responsible for MHCII degradation and inhibition of antigen presentation (54). Treatment with inhibitors of CPs induced a shift from a Th2 to a Th1 response in BALB/c (49) and prevented the activation of TGF-β in a macrophage cell line in vitro (221). CPB deficient L. mexicana did not affect the parasite infectivity of macrophages in vitro, but reduced their infectivity in BALB/c mice in vivo (3, 58), C3H and C57BL/6 mice were able to heal infection with these parasites (26). Recently, L. mexicana was found to inhibit IL-12 production by macrophages via using CP to degrade Iκα, Iκβ, and NFκβ, as well as inducing cleavage of JNK and ERK (29). CP inhibitors were found to reduce both L. major promastigote (in culture) and amastigotes intracellular growth (in macrophages) (217). These results show that CP have more significant role to L. major survival than L. amazonensis although CP function in L. amazonensis virulence.

2. Macrophages

Macrophages are part of the innate immune defense that recognizes foreign particle and non-self antigen by their surface receptors and subsequently engulf these particles by phagocytosis. Macrophage cells then mediate destruction and elimination of the pathogen. Leishmania has adapted to live inside the macrophages and resist these killing mechanisms.
2.1 Macrophage receptors

2.1.1 Mannose receptor:

Mannose receptor is a membrane glycolipid expressed in high levels on tissue macrophages. It binds sugars including l-fucose, D-mannose, and D-galactose, which are more common on the surface of microorganisms (229). The expression of this receptor is regulated, in part, by immunoglobulin receptors (213) and cytokines. For example mannose receptor is up-regulated by IL-4 (231) and IL-13 (57) but downregulated by IFN-γ (92). Ligation of the receptor results in the release of lysosome enzymes in culture (179), production of reactive oxygen intermediates (17), cytokines (218, 230) and upregulation of Fc receptors (171).

Different *Leishmania* species were found to use mannose receptor for invasion of macrophages including *L. donovani* (35, 36, 38, 257), *L. amazonensis* (196) and *L. mexicana* (187).

2.1.2 Complement Receptors (CR)

The complement system is activated by three pathways; 1) classic pathway which is dependent upon antibody and the first complement components (C1q, C1s, and C1r), 2) alternative pathway activated by microorganism directly and binds C3 on the surface of the pathogen and 3) the lectin pathway which requires mannose binding protein (MBP). Macrophage complement receptors include CR1 (CD35), CR3 (Mac1, CD11b/CD18) and CR4 (CD11c/CD18) all of which have been implicated in *Leishmania* macrophage invasion (66). CR1 is a monomeric receptor that bind C3b and C4b (128). Although it has been shown that *Leishmania* promastigotes opsonized with C3b are internalized by macrophages through
this receptor (46, 66, 67), a minimal role was found for CR1 in internalizing *L. amazonensis* (166).

CR3 is a heterodimer that binds to iC3b. *Leishmania* was reported to bind CR3 either by opsonization with iC3b or through direct binding of the receptor to gp63 and LPG on the surface of promastigotes (88, 124, 167, 207, 238). Although both *L. major* and *L. amazonensis* promastigotes were shown to bind to CR3 (167), only a minimal role for this receptor was reported in *L. amazonensis* (166) and *L. mexicana* (187) amastigote internalization. CR4 was found to bind to a lesser extent to both *L. major* and *L. amazonensis* (5).

### 2.1.3 Scavenger receptor

Scavenger receptor binds to and internalizes both lipoproteins and lipopolysaccharides (LPS). There are two members of this receptor family that have been shown to bind microbes; Scavenger receptor A (SR-1) and MARCO (macrophage receptor with collagenous structure) (186). There are no reports for the involvement of these receptors during *Leishmania* internalization. Although apoptotic cells were found to be internalized through this receptor leading to enhance *Trypanosoma cruzi* growth inside the macrophages (74). This receptor might play a role in *Leishmania* internalization in vivo.

### 2.1.4 Lectin-like receptors

There are wide varieties of surface lectins that mediate detection of self- and foreign-carbohydrates including hexoses, glucose, fucose, and mannose (253). These receptors were found to be responsible for 50% of the binding of *L. major* amastigotes and promastigotes to
resident macrophages, but only 10% of the binding to activated macrophages (165). The role of lectin-like receptors in binding *L. braziliensis* and *L. donovani* promastigotes to macrophages was demonstrated in the absence of serum (19, 100).

2.1.5 Fc receptors

FcR are a group of receptors which recognize the Fc portion of immunoglobulins each antibody class has their own specific receptor. FcγR binds IgG, FcαR binds IgA, FcεR binds IgE, FcμR binds IgM, and FcδR binds IgD. FcγR is further divided according to the isotype of IgG that is recognized by the receptor. FcγRI binds IgG2a, FcγRIIb or FcγRIIa only in humans, binds IgG1, and FcγRIII binds both IgG1 and IgG2a (106). Two forms of Fc receptors are found, soluble forms and membrane bound forms (47). Soluble forms are a result of proteolytic cleavage of FcγRII and III or through alternative splicing and have been reported to cause an inhibition of B cell proliferation and immunoglobulin production. Soluble forms also are thought to decrease antigen presentation in macrophages and DCs (75, 77). The FcR also divided according to their affinity of antibody binding: low affinity (FcγRII and FcγRIII) and high affinity (FcγRI, FcεRI, and FcαRI). Both low and high affinity receptors induce FcR aggregation, but the difference between the two types is just in the order of interaction. Low affinity receptors need antibodies to complex with antigen first then bind to the receptors through low avidity, while high affinity receptors bind the monomeric antibodies first and then complex with antigen which leads to receptor aggregation (47, 198).
After Fc receptors bind antibody and aggregate they transmit downstream signals to either activate macrophage function through the cytoplasmic signaling domains of the receptors termed immunoreceptor tyrosine-based activation motifs (ITAMs) associated with either the γ or ζ chain, or they inhibit macrophage function through immunoreceptor tyrosin-based inhibitory motifs (ITIMs). ITAM containing Fc receptors include FcγRI, FcγRIII, FcεRI, and FcαRI. Only FcγRIIb contains an ITIM motifs despite this, it the most abundant Fc receptor expressed on cells (106). Signaling through ITAM motifs results in endocytosis, phagocytosis, antibody dependent cellular cytotoxicity (ADCC) and production of inflammatory mediators, while signaling through ITIM motifs inhibits all of these activities (183).

Phagocytosis through Fc receptors requires a Fcγ chain (52). Phagocytosis of antibody-coated particles was abolished in macrophages deficient in Fcγ chain (237), but FcγRIIb missing the γ chain was shown to mediate phagocytosis of opsonized particles in macrophages and CHO cells transfected with FcγRIIb (96, 118).

*Leishmania* has been shown to use the Fc receptor to bind to and invade macrophages after antibody opsonization through phagocytosis (88, 99, 126, 166, 187). The necessity for Fc receptor in *Leishmania* persistence was demonstrated in Fcγ-receptor deficient mice and antibody deficient mice (27, 44, 126, 158). The mechanism *Leishmania* Fc receptors use for intracellular survival remain under debate, as it has been shown that in the absence of Fc receptor there is less phagocytosis and less recruitment of immune cells to the site of infection. This may be due to decreased antigen presentation (44), other reports have showed a role for Fc receptor and antibody opsonization in IL-10 production and subsequent
suppression of macrophage activation (27, 122, 158). Taking in consideration the differences between *Leishmania* species and the variant mouse strains used in these experiments, these results emphasize the fact that *Leishmania* uses these receptors for invasion and survival. Fc receptor signaling is not only a source of host-immune response resistance for the parasite but also could be as a source for macrophage activation and control *Leishmania* infection (50).

2.2 Phagocytosis

After foreign antigen binds a macrophage receptor, a clustering of the receptors lead to localized actin polymerization at the site of particle phagocytosis (85). Recruitment of receptors to the site of particle binding is required for actin dependent engulfment of the particle (129). After phagocytosis and vacuole formation, the vacuole undergoes maturation to form a phagolysosome. The first step in endosome maturation is formation of an endosome (early endosome), which is recognized by the presence of Ras-associated GTP-binding protein 5 (Rab5) and early endosome antigen 1 (EEA1). From there, the endosome is directed to form a recycling endosome expressing Rab11 or directed to mature into a late endosomes responsible for degradation. Late endosomes are acidic (pH 5.5) and have of Rab7, Rab9, mannose 6-phosphate receptor and lysosomal-associated membrane proteins (LAMP) expression. The final step of maturation is the formation of lysosomes, which are more acidic (pH< 5.5), with active lipases and proteases like cathepsin D and they contain LAMPS (247).

*Leishmania* live within parasitophorous vacuoles (PVs) inside the macrophages which vary in the size dependent on the species of the parasites. *L. amazonensis* and *L. mexicana* live in large communal PVs, whereas *L. major* and *L. donovani* live in tight
individual PVs (7). PVs are intracellular compartments surrounded a membrane enriched with late endosomal/lysosomal markers including Rab7, macrosialin, LAMP1, LAMP2 and vacuolar H+-ATPase. A poor fusion between the phagosome and lysosome in macrophages infected with *L. donovani* promastigotes was found due to LPG effect (59), however, both *L. amazonensis* and *L. major* were reported to induce phagosome and lysosome fusion within 30 min to form late endosomes. *L. amazonensis* amastigotes were found to induce the large PVs in much shorter time than promastigotes (45), as shown in previously (discussed in PPG section) these large PVs are thought to be due to the sPPG.

2.3 Macrophage killing mechanisms

2.3.1 Respiratory burst:

Respiratory burst is a potent antimicrobial response induced by the formation of reactive oxygen species intermediates (ROS) (63). Different sources for ROS are reported including; iNOS, NADPH oxidase, cyclo/oxygenase, lipoxygenase, xanthine oxidase, and the mitochondrial electron transport chain (70). Under inflammatory conditions the main sources of ROS are NADPH oxidase and xanthine oxidase (206). NADPH oxidase has five protein subunits (gp91, p22, p40, p47 and p67), that are assembled on the inner surface of the plasma membrane upon activation and react with NADPH as a substrate which in turn reduces oxygen to superoxide (48, 219). After superoxide is produced, it spontaneously, through enzyme catalysis, produces hydrogen peroxide, hydroxyl radicals, hypochlorous acid and peroxynitrite (in the presence of NO) (115). Both IFN-γ and TNFα are important for priming NADPH oxidase (65, 212, 242). Cross-linking of FcγRI can also mediate the activation of
NADPH oxidase through protein kinase C (PKC) cell signaling via FcR ITAMs motifs and Syk, a protein tyrosine kinase, Phosphatidylinositol 3-kinase (PI3K), phospholipase (PLC) and the release of Ca\(^{2+}\) (211, 260).

Superoxide is also produced by xanthine oxidase, which an enzyme produced by a wide range of cells with three active sites; flavin adenine dinucleotide (FAD), molybdenum (Mo) and cytochrome with haem iron. Xanthine oxidase can act as a peroxynitrite synthase as it has been shown to produce both nitric oxide by reducing nitrite to nitric oxide and superoxide by donating an electron from xanthine to oxygen. These two reactive compounds NO and superoxide, react together to produce peroxynitrite (159). Xanthine oxidase has been shown to be upregulated by various macrophages activation molecules; IFN-γ, TNF-α, IL1 and LPS (93).

2.3.2 Nitric Oxides (NO)

Inducible nitric oxide synthase (iNOS) is the enzyme responsible for NO production during an activated immune response and the presence of Ca\(^{2+}\) and calmodulin are needed for iNOS activity (258). L-arginine is the substrate from which iNOS obtains nitrogen to produce NO (101, 176). Macrophage activation with either IFN-γ or LPS induces the uptake of arginine and the upregulation of iNOS (28, 91). NO is microbicidal through different mechanisms including; protein nitrosylation, tyrosine nitration, inhibition of respiratory chain through inhibition of cytochrome oxidase, iron deprivation through binding of iron to an iron response element (IRE), inhibition of ribonucleotide reductase (RNR) which inhibits DNA synthesis, guanine oxidation that leads to DNA mutation, DNA strand degradation, DNA deamination and lipid oxidation (reviewed by (51)).
The importance of NO in *Leishmania* infection was demonstrated by the experiments in iNOS deficient mice which have exaggerated non-healing lesions after *L. major* infection (61, 250, 62). The same results were also obtained with use of an iNOS inhibitor (140, 232). Although the actual role for NO and iNOS for control of *Leishmania* infection is not clear, but it has been suggested that NO is cytostatic to *Leishmania* whereas peroxynitrite is cytotoxic (142).

### 2.3.3 LOHA (N⁶-hydroxy-L-arginine)

LOHA is produced as an intermediate product after iNOS react with arginine to produce citrulline and NO. LOHA has been shown to inhibit arginase, an enzyme used by *Leishmania* to produce ornithine and urea from arginine. This inhibition of arginase prevents polyamine synthesis essential for *Leishmania* growth and as a consequence LOHA was shown to control *L. major* and *L. infantum* growth (114).

### 2.3.4 Iron deprivation

Iron deprivation is used by host macrophages to control microbial infection (251). Many different mechanisms promote iron deprivation including inhibition of ferritin, a protein used to store iron by the cells, binding of iron lactoferrin, a protein that bind iron at high affinity in low pH, and iron binding to transferring, a protein produced in activated macrophages to bind intracellular iron (254, 69, 178). Treating macrophages with LPS and IFN-γ induces NO production and activates iron regulatory protein (IRP) to repress ferritin translation through reducing intracellular iron stores and decreasing iron availability (200).
2.3.5 Tryptophan degradation

Tryptophan is the least available amino acid for metabolism (181). Indoleamine 2,3dioxygenase (IDO) is an enzyme found in tissue which degrades tryptophan. IDO was found to be induced by IFNγ and augmented by TNF-α or LPS (255). There is no direct link between tryptophan degradation and the control of *Leishmania* to date although addition of external tryptophan increased *Leishmania* replication (172).

2.4 *Leishmania* evasion of macrophage activation

2.4.1 Antigen presentation and co-stimulatory molecule

Antigen presenting cells (APC) including macrophages, DCs, and B cells present antigen on surface of major histo-compatibility complex class II (MHCII) receptors to T cells inducing the T cells to become activated and proliferate. For Th1 cells to induce macrophages to control intracellular pathogen infection proper co-stimulatory molecule expression is necessary. These receptor ligand interactions include co-ligation such as CD40
with CD40L (on T cells) (20). L. donovani was found to suppress the expression of MHCII (201) while both L. amazonensis (192) and L. major (76) suppress antigen loading on MHCII. L. amazonensis amastigotes were found to internalize and degrade MHCII by parasite cysteine peptidases (54). The parasite has also been shown to sequester antigen within the phagolysosome thereby preventing antigen presentation to CD4* T cells (127). CD40/CD40L interactions were found to be necessary for control of L. major infection in a murine model of cutaneous disease (31, 224). L. donovani induced a five fold suppression of CD40 expression (25) which caused secondary inhibition of macrophage activation.

2.4.3 Modulation of cytokine secretion

2.4.3.1 Inhibition of IL-12 production

IL-12 is a critical cytokine for induction of a Th1 immune response through T cell activation and induction of IFN-γ production. BALB/c mice, susceptible to L. major infection, become resistant after treatment with IL-12 (98, 233). Mice resistant to L. major became susceptible when IL-12 was depleted with mAb (149). Infection with either L. donovani, L. major, L. mexicana and L. amazonensis was found to suppress IL-12 production (32, 252, 15, 117).

2.4.3.2 Induction of IL-10 and TGF-β

IL-10, a cytokine produced by a wide range of immune cells has been shown to suppress Th1 responses and augments Th2 mediated immune responses. IL-10 inhibits macrophage activation, antigen presentation, co-stimulatory molecule expression and
production of IL-12 and TNF-α (163). *L. donovani* was found to induce IL-10 mRNA expression 16.5 fold in infected macrophages (156). Ligation of FcγR were found to induce IL-10 production in both *L. major* and *L. mexicana* infection (27, 122, 158), and IL-10 deficient mice were resistant to infection with either *L. major* or *L. mexicana* (27, 177). However IL-10 deficient mice infected with *L. amazonensis* were not found to be different from wild type mice in lesion size or parasite load (119), indicating that although *L. amazonensis* and *L. mexicana* are related, immune responses to these two species of *Leishmania* are different and IL-10 does not play a role in *L. amazonensis* persistence.

TGF-β is another immunoregulatory cytokine shown to inhibit macrophage activation as well as IL-2 and IFN-γ production from T cells (64, 243). *L. amazonensis* was found to increase TGF-β production through exposing phosphatidylserine (PS) on the amastigotes surface which binds a ligand on the macrophage and triggers TGF-β secretion (53). Different *Leishmania* spp. were reported to induce TGF-β production to evade macrophage activation including: *L. amazonensis, L. tropica, L. chagasi, L. donovani* and *L. major* (241, 78, 138, 12, 13, 199). TGF-β similar to IL-10 as it is used by the immune system as negative feed back to regulate the immune response, *Leishmania* spp. use these cytokines to attenuate the immune system and evade macrophage killed.

2.4.4 Intracellular signaling

2.4.4.1 Activation of cellular phosphatases

Small heterodimer partner-1 (SHP-1) is most prominent regulated phosphatase in macrophages. SHP-1 is a protein tyrosine phosphatase (PTP) that is been described as a
negative regulator of tyrosine phosphorylation and signaling transduction (174). *L. major* and *L. donovani* infected mice treated with a PTP inhibitor demonstrated enhanced control of infection, as evident by a significant reduction in *L. major* lesion size, and complete protection against *L. donovani* (180). SHP-1 deficient mice did not develop lesions and when low numbers of parasites were recovered they were infected with *L. major* (73). This suggests that *Leishmania* activates cellular phosphatases to inhibit macrophage activation.

### 2.4.4.2 Inhibition of Protein Kinase C (PKC)

PKC is related to a family of serine/threonine kinases which participate in several cell-signaling pathways including phagocytosis through CR3 and FeR, phagolysosome fusion, oxidative burst and induction of inflammatory mediators (164, 261, 139). *Leishmania* spp. have been reported to inhibit PKC (228, 105). LPG, GIPL and GPI have all been implicated as possible parasite molecules involved in this inhibition (40, 18, 236, 155).

### 2.4.4.3 Mitogen-activated protein Kinase (MAPK)

The MAP kinases are a family of kinases responsible for activating many different macrophage functions. This family is composed of ERK, p38 and JNK/SAPK (43). The effect of *Leishmania* infection on the MAPK activation varies depending on the species and stage of the parasite and the cell type used for infection. *L. amazonensis* amastigotes were found to inhibit ERK1 induction in peritoneal macrophages through *Leishmania* PTP (145), *L. donovani* and *L. major* attenuated phorbol myristate acetate (PMA) induced MAPK (175, 87). A study in which macrophages are infected with *L. mexicana* amastigotes showed degradation of ERK and JNK but not p38 MAPK by parasite cystine peptidase (30). In contrast, LPG from
*L. donovani* was found to induce the activation of the three MAPK (10), while infection itself inhibited ERK activation through ceramide, sphingolipid precursor molecule (80). Another group recently showed a weak CD40 signal from macrophages infected with *L. major* promastigotes caused ERK phosphorylation and IL-10 production which inhibits further macrophage activation (146). *Leishmania* spp. appear to manipulate MAP kinases either by activation or inhibition in a manner that would help parasite survival within the macrophages.

### 2.4.5 Peroxidoxin

Peroxidoxins, also known as peroxiredoxins and thiol-specific antioxidants, are enzymes that detoxify hydrogen peroxide, hydroxy radicals and peroxynitrite (24). *L. major*, *L. infantum* and *L. chagasi* have been shown to produce peroxidoxins (137, 33, 11). Peroxidoxins are produced by *Leishmania* spp. to protect themselves from RNS and ROS damage produced by macrophages and therefore evade macrophage killing.

### 2.4.6 Superoxide dismutase (SOD)

SOD is an enzyme produced by the cells to protect themselves from the superoxide they produce during oxidative burst. This protection is mediated by dismutating superoxide to form O₂ and H₂O₂. Several *Leishmania* spp. have been shown to produce SOD themselves including *L. chagasi*, *L. tropica* and *L. donovani* (157, 170, 184, 81). SOD-deficient *Leishmania* have reduced survivability inside macrophages and more sensitivity to hydrogen peroxide in culture (81). SOD produced by *Leishmania* is one of the mechanisms where the parasite avoids superoxide cytotoxicity.
2.4.7 Glutathione

Glutathione is a thiol produced by both host cells and *Leishmania* parasites. Both host cells and parasite produced glutathione plays a role in detoxifying NO and protecting *Leishmania* from NO cytotoxicity (204).

3. Host immune response to *Leishmania*

A protective immune response to *Leishmania* infection is mediated through induction of productive cell-mediated immune response. Many cell types have been reported to participate in this immune response against *Leishmania* infection both negatively and positively. The primary cells types described as inducers of macrophage activation are CD4+ T cells and to a lesser extent CD8+ T cells. Antigen presenting cells (APCs) and in particular dendritic cells are necessary for antigen specific T cells to be activated and proliferate. Two phenotypes of CD4+ T cells have been identified; T helper 1 (Th1) and T helper 2 (Th2). C3H, C57BL/6 and C57BL/10 mice mounting a Th1 response, characterized by IL-12, TNF-α, and IFN-γ production, heal *L. major* infection. BALB/c mice that mount a Th2 response, with high levels of IL-4, IL-10, and IL-13, develop an exaggerated non-healing lesion when infected with *L. major* (248).

3.1 CD4+ T cells and *L. major* infection
CD4+ T cells are the primary source of IFN-γ, which is known to activate the macrophages to kill intracellular parasites (97). Mice deficient in IFN-γ or treated with antibodies against IFN-γ cannot heal L. major infection and subsequently mount a Th2 response (132, 214, 249). When IFN-γ is injected into susceptible mice the mice develop smaller lesions and mount a Th1 response (239, 214). Other cytokines including IL-12, produced by DCs, macrophages, neutrophils, and B cells, induce CD4+ T cells to produce IFN-γ and induce a Th1 response (240). The receptor for IL-12 has two subunits IL-12Rβ1 which binds IL-12 and IL-12Rβ2 which is responsible for signaling transduction in the cell (240). Mice lacking either IL-12 or the IL-12 receptors cannot heal L. major infection and usually mount a Th2 response (149, 148, 185, 34).Injecting IL-12 into susceptible mice at the time of L. major infection can induce healing with a shift to IFN-γ production and a Th1 response (98, 185, 233). In addition to IL-12 activation and MHCII presentation, costimulatory molecules including CD28 expressed on T cells and their ligands B7.1 and B7.2 expressed on DC and macrophages play an important role in CD4+ T cell activation (37). T cell expression of CD40 ligand which binds to CD40 on DCs has been found to play a crucial role in IL-12 production from the DC population (86). Direct contact between the CD4+ T cells with macrophage MHCII were not necessary for activation of macrophages and control of L. major in vivo (135).

CD4+ T cells are responsible for production of Th2 cytokines including IL-4, IL-10, produced also from macrophages, and IL-13. In susceptible mice, a population of CD4+ T cells that are Vβ4 Vα8+ and recognize L. major LACK antigen was shown to be responsible for IL-4 production (133). Early IL-4 production directs the immune response toward a Th2
response by inhibiting IL-12 production and downregulate IL-12Rβ2 expression on T cells (131), (102), (133). Susceptible mice treated with an anti-IL-4 antibody heal *L. major* infection (39, 143, 210). IL-10 is known to inhibit macrophage activation and susceptible mice lacking IL-10 or depleting IL-10 with anti-IL-10 antibody will heal *L. major* infection (177, 122).

### 3.2 CD4⁺ T cells and *L. amazonensis*

Contrary to the Th1/Th2 immune response paradigm defined by *L. major* for determining resistance or susceptibility to infection, the susceptibility of mice to *L. amazonensis* has been shown to be a result of an absence of either Th1 or a Th2 response as significantly low levels of both IL-4 and IFN-γ are detected after infection (1, 116, 120). Additional support for this assessment is that IL-4 deficient C57BL/6 mice remain susceptible to *L. amazonensis* infection. In these mice, IL-12Rβ2 suppression was detected; obviously independent of IL-4 mediated suppression (120). IL-10 deficient mice also cannot heal *L. amazonensis* infection (119, 182). Although anti-TGFβ or anti-IL-4 also induces a reduced lesion size of infected BALB/c mice, high parasite load was still detected in the lesion (12, 2). Together these results indicate a minimal role for Th2 cytokines in promoting susceptibility to *L. amazonensis* infection therefore suggesting the role of alternative immune evasion strategies utilized by this parasite to survive intracellular macrophages killing.

When RAG-2 deficient C57BL/6 mice that contain no T cell are infected with *L. major*, they develop exacerbated lesions (160), while infection of these mice with *L. amazonensis* induces only a very small lesion with a very low number of parasites present in
the lesion (223). A recent study showed increased replication of *L. amazonensis* amastigotes in the presence of IFN-γ (194). This indicates not only is the immune response to *L. amazonensis* infection reduced in its Th1 response, but the parasite uses Th1 cytokines specifically to promote virulence in vast contrast to the situation during *L. major* infection.

3.3 B cells

B cell function in the immune system is a reflection of the humoral immune system and previously thought to primarily reflect a Th2 response, although B cells role in cell mediated immunity is not neglected. In addition to B cells role in secreting antibody, they are an APC through using MHCII and secret multiple cytokines including IL-12. The role of B cells in *Leishmania* infection has been studied using different mouse strains and different *Leishmania* species with various results for their specific role as APC or the role of secreted antibodies from B cells.

Contradictory reports for the role of B cells in *L. major* infection range from B cells being not necessary to necessary for healing of infection. B cells were reported to be not necessary for healing in SCID mice reconstituted with CD4+ T cell from healed *L. major* infection (245, 4). Infecting B cell-deficient BALB/c or C57BL/6 mice with *L. major* did not have a different disease outcome from wildtype mice (44, 23). Other reports indicate a role for B cells in *Leishmania* susceptibility including studies using BALB/c mice lacking B cells (104, 209) or a study which converted T cell-reconstituted, resistant SCID mice to susceptible after co-transfer of B cells (103). Only in one study using C3H mice B cells were found to be necessary, where B cells were shown to be needed to induce proper T cell generation and healing of the infection (215). The B cells role is obviously linked to the role
of antibody in *Leishmania* virulence. This is discussed in the Fc receptors section of the introduction.

The role for B cells during infection with *L. mexicana* complex parasites, *L. mexicana*, *L. amazonensis*, and *L. pifanoi*, is linked to the necessity for antibody for parasite invasion as discussed previously in the Fc receptor section (44, 126). Opsonized *L. mexicana* induces IL-10 which further suppresses macrophage activation (27). In summary, these studies showed mostly either a negative or no role for B cells in protective immune response to *Leishmania* infection.

4. General conclusion

Despite the fact that the main role of macrophages in the immune system is to engulf pathogens and kill them, parasites such *Leishmania* manage to survive and live inside these macrophages. *Leishmania* has developed multiple different strategies to survive within the host. Some of these strategies are unique to each *Leishmania* spp., even within genetically related species, e.g. with the *L. mexicana* complex there are variations in the parasites interaction with the host immune system. As an example of these strategies, macrophages are equipped with surface receptors to detect foreign antigen, *Leishmania* use this system via surface molecules which allow the parasite to be detected and engulfed quickly and hide inside the macrophages and thereby to evade being killed by the complement system. Surface molecules of *Leishmania* change with the developmental stage of the parasite, as they need to be protected from the harsh environment inside the insect gut and from the first contact with
the blood, promastigotes have specific surface molecules that help them attach to the insect gut and the same time prevent their lysis by serum. Amastigotes have fewer surface molecules which specifically prevent activation of macrophages while reproducing inside the mammalian host.

Macrophages need to be activated to kill intracellular Leishmania. This activation is linked to the production of Th1 cytokine including IFNγ which induces macrophages to secrete digestive enzymes, RNS, and ROS to kill Leishmania. Many species of Leishmania have developed means to induce a Th2 response and inhibit a Th1 response, as seen during L. major infection. L. amazonensis induces a different immune response where there is an absence of either a Th1 or Th2 responses. Even when a Th1 response is induced, L. amazonensis parasite still resists macrophage activation. This indicates the complexity of L. amazonensis mechanism to resist macrophage activation and killing in the environment where other Leishmania spp. are killed. This macrophage survival mechanism of L. amazonensis may explain why L. major infection is usually a self-limiting disease while L. amazonensis produces a persistent chronic lesion. The understanding of this resistance by L. amazonensis to macrophage killing compared to L. major is the center of the work many research groups and will be the focus of this thesis.
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CHAPTER 2

Macrophage killing of *Leishmania amazonensis* amastigotes requires both nitric oxide and superoxide

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ABSTRACT

The lack of understanding of the requirements for effective and efficient intracellular *Leishmania amazonensis* killing by activated macrophages has been a barrier to vaccine development against this parasite. Here we report using inhibitors of superoxide and inducible nitric oxide synthase that the presence of both superoxide and nitric oxide is necessary for killing of *L. amazonensis* amastigotes within LPS/IFN-γ activated bone marrow-derived macrophages generated from C3H mice. Nitric oxide alone was sufficient to control *L. major* infection, as addition of a nitric oxide donor was sufficient to control *L. major* but not *L. amazonensis* infection. Addition of an extracellular signal-regulated kinase (ERK) inhibitor to *L. amazonensis*-infected macrophages increased the ability of these activated macrophages to kill *L. amazonensis* amastigotes. This enhanced macrophage killing through addition of ERK inhibitor was abrogated by inhibition of superoxide or iNOS, indicating that ERK activation may modulate effective macrophage killing lending to the ability of *Leishmania amazonensis* to resist elimination within activated macrophages.
Keywords: *Leishmania amazonensis*, macrophage, superoxide, nitric oxide, ERK

INTRODUCTION

Upregulation of inducible nitric oxide synthase (iNOS) has emerged as being important for leishmanicidal activity of infected macrophages. Evidence indicates that the anti-parasitic activity of this enzyme involves generation of reactive nitrogen intermediates; nitric oxide and/or other reactive nitrogen molecules. These reactive nitrogen species may, in turn, interact with NADPH-oxidase dependent superoxide to produce or enhance cytotoxic responses (reviewed in). Recent reports suggest that the contribution of NADPH-oxidase-dependent superoxide production to a leishmanicidal response may vary between different cell types and stage of infection. In addition to nitric oxide production, the iNOS generated arginine metabolite N\textsuperscript{ω}-hydroxy-L-arginine (LOHA) has been shown to limit the growth of *Leishmania* within infected macrophages and play a significant role in limiting *in vitro* infection rate.

Host intracellular signaling pathways are specifically targeted by the parasite during *Leishmania* infection to prevent a productive immune response (reviewed in). Contradictory reports were shown for the role of mitogen-activated protein (MAP) kinases in the resistance of *Leishmania* to macrophage activation. As *L. mexicana* was shown to inhibit IL-12 production through extracellular signal-regulated kinase (ERK) degradation, both *L. amazonensis* amastigotes and *L. donovani* promastigotes were found to prevent ERK phosphorylation. On the other hand, *Leishmania* phosphoglycans were found to inhibit macrophage activation through ERK activation. *L. major* promastigote lipophosphoglycan
(LPG) has been shown to inhibit macrophage IL-12p40 production through extracellular signal-regulated kinase (ERK) activation. L. major-infected macrophages produce limited CD40-induced expression of IL-12p40 as a result of enhanced IL-10 production mediated through the host ERK pathway. However, LPG from L. donovani were found activate activation protein 1 (AP-1) through ERK. A variety of Leishmania spp. including L.
amazonensis (reviewed in 14, 18) have been shown to inhibit and/or resist macrophage activation, but the requirements for effective intracellular L. amazonensis killing by activated macrophages and the mechanisms involved remain unknown.

We have focused our studies of L. amazonensis infection by using the C3H mouse strain as these mice are susceptible to infection by this parasite and yet resistant to the related parasite L. major. In particular, we have found that a previous infection with L. major limits disease upon subsequent infection with L. amazonensis indicating that C3H mice upregulate an effective anti-L. amazonensis immune response if activated appropriately. In this current work, we examined the capability of L. amazonensis amastigotes to prevent macrophage activation compared to L. major amastigotes infection in vitro by infecting first then induce the activation. In this system, both LPS and IFNg were chosen as a potent macrophage activator, as presence of IFNg prevents L. amazonensis inhibition of LPS induced iNOS. Under these defined pro-activation conditions, L. amazonensis amastigote parasites were not killed as efficiently as L. major amastigotes despite equivalent iNOS and nitric oxide levels. Unlike L. major, L. amazonensis was resistant to inhibition of replication mediated by LOHA, although replication of L. amazonensis was not a major factor in the ability of this parasite to persist within activated macrophages. L. amazonensis amastigotes were also shown to be resistant to the potentially leishmanicidal effects of the nitric oxide donor.
(SNAP), indicating that although nitric oxide is sufficient to control \textit{L. major} amastigote infection, additional factors are required for intracellular macrophage killing of \textit{L. amazonensis} amastigotes. Addition of the ERK inhibitor PD98059 to activated macrophages promoted killing of \textit{L. amazonensis} amastigotes, dependent upon the production of superoxide. ERK activation, therefore, may be a critical part of host cell signaling changes derived by intracellular infection with \textit{Leishmania amazonensis} leading to an inadequate accumulation of multiple reactive oxygen species and promote intracellular parasite survival. These findings highlight the differences required for intracellular killing of disparate \textit{Leishmania} species confirming that the definition of an adequate host immune response against one \textit{Leishmania} species does not apply to all parasites of this genus.

**MATERIAL AND METHODS**

**Mice.** C3HeB/FeJ and C3Smn.CB17-Prkda^{scid}/J (C3H scid) mice were obtained from Jackson Laboratory (Bar Harbor, Maine). Mice were maintained in a specific-pathogen-free facility. For the propagation of lesion derived amastigotes C3H SCID mice were infected with 5x10^6 stationary-phase promastigotes in 50 \( \mu l \) of PBS in the left hind footpad. All procedures involving animals were approved by the Committee on Animal Care at Iowa State University.

**Parasites.** \textit{Leishmania major} (MHOM/IL/80/Freidlin) and \textit{Leishmania amazonensis} (MHOM/BR/00/LTB0016) promastigotes were grown to stationary phase in Grace’s insect culture medium (Technologies, Gaithersburg, MD) with 20\% heat-inactivated FCS, 2 mM glutamine, 100 U penicillin per ml, and 100 \( \mu g \) of streptomycin per ml. Amastigotes that
were used for in vitro infection were tissue derived and were harvested from lesions of C3H SCID mice as described 16.

Cells and cell culture. Cells were obtained from the bone marrow of the mouse femur and tibia (15-20 x 10^6 cells) and plated in a 150 x 15 mm Petri dish with 30 ml of macrophage medium containing 30% L-cell conditioned medium, 20% FCS and 50% Dulbecco's modification of Eagle's medium (DMEM), 2 mM glutamine, 100 U penicillin per ml, 100 μg of streptomycin per ml, and 1 mM sodium pyruvate at 37 °C and 5% CO₂. After 2 days another 20 ml of macrophage medium was added to the Petri dish. At day 7 the non-adherent cells were removed and the plate scraped to harvest the adherent cell population. After washing with PBS, live cells were counted using trypan blue exclusion and resuspended in complete tissue culture medium (CTCM) containing DMEM, 10% FCS, 2 mM glutamine, 100 U penicillin per ml, 100 μg of streptomycin per ml, 25 mM 4(2-Hydroxyethyl)piperazine-1-ethane sulfonic acid (HEPES), and 0.05 μM 2-β-mercaptoethanol.

Macrophage infection and activation. BMM were plated in 24 well plates with glass cover-slips at a rate of 5×10^5 cells/well, in 1 ml CTCM. After 24 h the BMM were infected with either L. major or L. amazonensis amastigotes at a 3:1 ratio and incubated at 34°C for 24 h. The wells were washed two times with DMEM to remove extracellular amastigotes and a final volume of 1 ml CTCM was added. The macrophages were then left non-activated or activated with 100U/ml IFN-γ and 100 ng/ml LPS (E. coli J5 from Sigma-Aldrich, St. Louis, MO). For replication inhibition experiments, a concentration of 500 μM N⁶-Hydroxy-nor-L-Arginine (LOHA, Calbiochem, La Jolla. CA) or 4 mM hydroxyurea (Alfa
Aesar, Ward Hill, MA) were used in each well of both activated and non-activated infected macrophages. Also, 15 μM Mn(III)tetrakis(4-benzoic acid)porphyrin chloride (MnTBAP, BIOMOL, Plymouth Meeting, PA), 100 μM S-Nitroso-N-acetylpenicillamine (SNAP, Sigma-aldrich, St. Louis, MO), 1 mM L-N6-(1-iminoethyl)-lysine (l-NIL, A.G. Scientific, San Diego, CA) or 20 μM 2'-Amino-3'-methoxyflavone (PD 98059, Alexis, Lausen, Switzerland) were added at the time of activation. The cover-slips were removed, dependent on the experiments, after 24, 48, and 72 h post-activation and stained using nonspecific HEMA 3 stain set (Fisher Diagnostics, Middletown, VA) and mounted on glass slides.

**Determination of the infection rate of macrophages and parasite count.** Each individual coverslip was counted via light microscopy by examining 3 areas at 1000x total magnification. In each area, 100 macrophages were examined and the number of infected macrophages and the number of parasites in each macrophage were counted. The average of the 3 areas was used to determine the percent of infected macrophages and the number of parasites per macrophage on each coverslip.

**Determination of nitric oxide.** Nitrite concentrations were determined using the Greiss reagent as described previously. Briefly, equal volumes (50 μl) of cell culture medium and Greiss reagent (LabChem, Pittsburgh, PA) were mixed and incubated at room temperature and the absorbance was measured at 550 nm with a microplate reader (Molecular Devices, Sunnyvale, CA). The nitrite concentration was determined using a standard curve generated with sodium nitrite.

**Western blot.** Twenty four hours after activation the macrophages were washed two times with 1ml PBS, then 100 μl of washing buffer was added (0.32 M sucrose, 3 mM
calcium chloride, 2 mM magnesium acetate, 0.1 mM EDTA, 10 mM Tris (Hydroxyethyl) aminomethane (Tris) (pH 8.0), 5 mM sodium fluoride, 1 mM dithiothritol (DTT), 0.5 mM phenylmethanesulfonyl fluoride (PMSF), 0.1 mM sodium orthovanadate and 20 μl per ml protease inhibitor cocktail (Sigma, St. Louis, MO). Then 100 μl of lysis buffer was added (wash buffer plus 1% nonidet P-40). The suspension was centrifuged at 2500 g and the supernatant was stored at – 80 °C. Protein concentrations were measured via BCA (bicinchoninic acid) protein assay (Pierce, Rockford, IL) using BSA as a standard. The cytoplasmic extracts were separated using 8% PAGE and transferred to nitrocellulose membrane using a semi-dry blotting apparatus from Bio-Rad (Hercules, CA). The membrane was blocked with 5% nonfat dry milk in PBS 0.1% Tween. Rabbit polyclonal anti-mouse iNOS (Upstate, Lake Placid, NY) was hybridized to the membrane overnight at 4 °C at a 1:5000 dilution. The membrane was then washed and hybridized to a secondary goat anti-rabbit conjugated to hydrogen peroxidase (Jackson ImmunoResearch, West Grove, PA) at a 1:50,000 dilution, and the signal was detected using the Pierce Supersignal Reagents (Pierce, Rockford, IL) as directed by the manufacturer. The membranes were rehybridized with a rabbit polyclonal anti-mouse actin Ab at a 1:500 dilution (Sigma, Saint Louis, Missouri) as described above.

**ELISA for IL-10.** The level of IL-10 protein in supernates was determined by ELISA according to the manufacturer's instructions (Pharmingen, San Diego, CA).

**Statistics.** Data analysis was performed by using Statview 5.0.1 (SAS, Cary, NC). Statistical significance was determined by either Scheffe's test for pair wise comparisons
when comparing between parasite species or a paired t-test when comparing treatment groups within a parasite species as indicated in the figure legend.

RESULTS

*L. amazonensis* amastigotes are resistant to killing by IFN-γ/LPS-induced macrophage activation. To determine whether there is a relative resistance of *L. amazonensis* amastigotes to killing by macrophages with a defined activation stimulus, BMM derived from C3H mice were infected first with *L. amazonensis* or *L. major* amastigotes and then stimulated 24 h later with LPS and IFN-γ. A disparate parasite survival response was detected three days post stimulation, as *L. amazonensis* maintained a high infection rate of 86% of non-activated-infected control compared to 56% for *L. major* (Fig. 1a). There were no significant differences in nitrite accumulation between *L. major* and *L. amazonensis*-infected, activated macrophages at any time point observed, indicating that nitrite production did not determine the difference in macrophage killing of *L. major* as compared to *L. amazonensis* (Fig. 1b). Western blot analysis confirmed equivalent iNOS protein expression in uninfected, *L. amazonensis* infected, and *L. major* infected macrophages at 24 h post-LPS/IFN-γ activation (Fig 1c).

The numbers of parasites per infected macrophage were counted to determine if macrophage activation influenced the *in vitro* intracellular growth of the *L. amazonensis* parasites. The results in Figure 1d show that under activation conditions both species had a significant reduction in the number of parasites per infected macrophage at day 3 post-
activation. However, in both the control and activated groups there were relatively more \textit{L. amazonensis} parasites per macrophage than corresponding \textit{L. major} infected cells (control = 9.82 ± 2.52 and 3.92 ± 0.88, respectively; activated = 4.7 ± 0.55 and 1.86 ± 0.12, respectively).

\textit{L. amazonensis} amastigotes are resistant to the growth inhibitory effects of LOHA, although replication plays a minimal role in \textit{L. amazonensis} amastigote survival \textit{in vitro}. Recent studies have demonstrated that for \textit{L. major} the infection rate of macrophages \textit{in vitro} was partially dependent on the ability of the parasite to replicate within the infected macrophages \textsuperscript{18}. Therefore \textit{L. amazonensis} persistence despite relatively high levels of nitrite production and iNOS expression could indicate resistance to growth inhibitory effects of LOHA \textsuperscript{7}. LOHA is an intermediate of NO generation by iNOS and an inhibitor of arginase activity. Arginase is an enzyme important for polyamine synthesis, a metabolic pathway necessary for parasite growth. We tested the hypothesis that \textit{L. amazonensis} is resistant to LOHA allowing increased replication inside macrophages. LOHA (500 µM final concentration) was added to cultures at the time of macrophage activation and the percent of infected macrophages and the numbers of parasites per infected macrophage were evaluated as previously described. \textit{L. major} was sensitive to the effects of LOHA (Fig. 2) as there was a significant decrease in the percent of macrophages infected with \textit{L. major} as well as a reduction in the number of parasites per macrophage in the presence of the inhibitor under non-activating conditions (Fig 2 a and b). Under activation conditions there was a significant effect of LOHA as determined by a further reduction in the macrophage infection rate, although as the average number of parasites per macrophage was close to one no change in the number of parasites per macrophage could be detected (Fig. 2 c and d). In contrast,
LOHA had no effect on either of these parameters in *L. amazonensis* infected macrophages under non-activated or activated conditions (Fig 2). These results indicate that *L. amazonensis* is resistant to LOHA inhibition of replication and suggest that enhanced *L. amazonensis* replication during macrophage activation may maintain a higher rate of infection in the *in vitro* assay. To then specifically test the effect of replication on the enhanced infection rate of *L. amazonensis* we used the DNA synthesis inhibitor hydroxyurea (4 mM final concentration). *L. major* survivability was again decreased, similar to the results seen with LOHA confirming a role for parasite replication in influencing the rate of macrophage infection with *L. major* in our *in vitro* system (Fig. 2). In contrast, although hydroxyurea did significantly reduce *L. amazonensis* replication, as seen as a decrease in the numbers of parasites per macrophage under non-activated conditions (Fig. 2b), this reduced replication rate had no effect on determining the percent of macrophages that remained infected with the parasite (Fig. 2a). In addition, although the hydroxyurea mediated inhibition of replication did reduce the percentage of *L. amazonensis* infected macrophages under activated conditions (12% reduction) (Fig 2c) the differences between *L. amazonensis* and *L. major* under identical conditions were still dramatic. Altogether these results indicate that replication of the parasite plays a significant role in the persistence of *L. major* under both activated and non-activated conditions, while in contrast, although *L. amazonensis* is resistant to the growth inhibiting effects of LOHA, enhanced replication of *L. amazonensis* is not entirely responsible for the ability of this parasite to persist in activated macrophages compared to *L. major*.

*L. amazonensis* amastigotes are resistant to NO. A previous study used the nitric oxide donor (SNAP) to investigate the effect of nitric oxide alone on *L. amazonensis* or *L.
major promastigotes within infected macrophages and showed that both parasites were susceptible to killing mediated via nitric oxide\textsuperscript{20}. Despite similar levels of nitrite (Fig. 1b), our studies demonstrate a differential susceptibility between the two parasites suggesting that \textit{L. amazonensis} amastigotes are more resistant to nitric oxide as compared to \textit{L. major} amastigotes. Treatment of amastigote-infected macrophages with SNAP (100 \textmu M) was sufficient to control \textit{L. major} infection (Fig. 3a), but \textit{L. amazonensis} remained resistant to SNAP with or without IFN\gamma and LPS activation (Fig. 3a). To determine if nitric oxide was playing any role in limiting the \textit{L. amazonensis} infection \textit{in vitro} we inhibited iNOS using l-NIL (1 mM). There was an abrogation of the \textit{L. major} killing and the \textit{L. amazonensis} infection rate was significantly enhanced over the non-activated control (Fig. 3b). After l-NIL treatment there was also an increase in the number of intracellular \textit{L. amazonensis} and \textit{L. major} parasites per macrophage (data not shown). These findings indicate that \textit{L. major} amastigotes are highly susceptible to NO produced by activated macrophages. In contrast, although NO contributes to some limitation of \textit{L. amazonensis} amastigote survival during activation conditions, it does not kill \textit{L. amazonensis} as efficiently as \textit{L. major}.

The ERK inhibitor PD98059 promotes superoxide dependent killing of \textit{L. amazonensis} in activated macrophages. IL-10 has been described as a cytokine that can inhibit macrophage activation and is produced in response to infection by \textit{Leishmania} parasites within infected macrophages under a variety of experimental conditions. To determine if higher levels of IL-10 were associated with the differential resistance of \textit{L. amazonensis} in activated macrophages, IL-10 levels in the supernatant of the cultures were measured by ELISA. No significant differences in the level of IL-10 between \textit{L.}
_amazonensis, L. major_ and uninfected macrophages were observed (data not shown).

Recently, _Leishmania_ has been shown to limit macrophage activation through activation of mitogen-activated protein kinase (MAP kinase) ERK $^{21, 13}$. Although the effects of ERK activation have been associated with increased IL-10 expression, in these studies, we wanted to test if the ERK signaling pathway could play a role in limiting macrophage responses during _L. amazonensis_ amastigote infection _in vitro_. Resistance of _L. amazonensis_ to macrophage killing was significantly diminished in the presence of the ERK inhibitor PD98059 (20 μM) and the reduction of the infection rate when compared to the control (52.57±8.01, Fig. 4) was similar to that of _L. major_ amastigotes within activated macrophages (Fig. 4). The ERK inhibitor alone did not affect the infection rate in non-activated macrophages for both parasites (data not shown). As we have demonstrated no difference in iNOS and NO microbicidal pathways between _L. amazonensis_ and _L. major_ in our _in vitro_ system, we hypothesized that other effector molecules were enhanced in the presence of PD98059. Therefore we co-treated the infected and activated cells with the cell permeable superoxide dismutase mimetic MnTBAP (15 μM) to scavenge superoxide or 1-NIL (1 mM) to inhibit iNOS. As seen in figure 4, enhanced macrophage killing of _L. amazonensis_ established by the ERK inhibitor, was negated in the presence of the superoxide inhibitor although there was not significant (p= 0.065) decrease in the killing of _L. major_, the infection rate was 67% of the uninfected control. Again, both species of _Leishmania_ killing were inhibited in the presence of iNOS with the ERK inhibitor. Neither MnTBAP nor 1-NIL alone has any effect on the macrophage infection rate in the absence of activation (data not
shown). Therefore, the ERK inhibitor PD98059 leads to an induction of superoxide and iNOS dependent killing of *L. amazonensis* parasites in infected macrophages.

**DISCUSSION**

Previous studies as well as the initial experiments shown here (Fig. 1) demonstrate a specific resistance of *L. amazonensis* parasites to macrophage-activated killing as compared to *L. major*\(^{20,22,23}\). We have extended these studies and the results support a model suggesting that *L. amazonensis* amastigote killing is dependent upon both nitric oxide and superoxide production from activated macrophages whereas *L. major* amastigotes are more susceptible to killing primarily by nitric oxide alone.

Other studies have demonstrated NO-dependent killing of *L. amazonensis* in activated macrophages, as determined by enhanced parasite DNA fragmentation\(^{24}\). However, the relative resistance of *L. amazonensis* amastigotes to IFN-γ/LPS mediated macrophage activation was not dependent on a parasite induced inhibition of the iNOS-NO pathway in our studies (Fig. 1b and c). The cellular and molecular targets of iNOS-mediated leishmanicidal activity are somewhat controversial and NO is not thought to act alone within the host macrophage during the leishmanicidal response. Inhibition of iNOS by 1-NIL increased the infection rate of activated macrophages for both parasites (Fig. 3b), agreeing with previous work demonstrating the role of iNOS in controlling *Leishmania* *in vivo*\(^{1,2,3}\) and *in vitro* (reviewed \(^{5}\)). The significant increase in the macrophage infection rate to 130% of non-activated control with the *L. amazonensis* amastigotes by 1-NIL could be consistent
with a recently identified role for IFNγ in enhancing parasite replication (if iNOS mediated NO production was inhibited (Fig. 3) 25.

Our results support the evidence that NO acts as a cytostatic agent against L. amazonensis. Other factors, including superoxide, are needed to produce peroxynitrite, which has been shown to be more potent in killing L. amazonensis amastigote parasites in vitro 4. In macrophages, peroxynitrite and its derivatives are thought to be generated primarily from the close association of NO and superoxide (reviewed in 5). The lack of effect by NO alone on L. amazonensis survival was contradictory to results shown by Gomes 20. This difference could be due to the use of a different dose of SNAP or to the different stage of the parasite (promastigotes vs amastigotes), macrophage type (peritoneal vs BMM) or mouse strain (CBA/J mice vs C3H).

Low levels of induced CD40 signaling within L. major infected peritoneal macrophages were found to cause ERK phosphorylation, IL-10 upregulation and inhibition of IL-12 and iNOS production 21. Here we show that the ERK inhibitor, PD98059, reduced the survivability of intracellular L. amazonensis in BMM via superoxide- and NO-dependent mechanisms (Fig. 4). The absence of differential expression of IL-10 in our system between L. major and L. amazonensis infection as determined by ELISA, was expected based on previously observed findings that IL-10 has been shown to inhibit production of both iNOS and NO 26,27. As neither iNOS nor NO are differentially produced between L. amazonensis and L. major infection, regulatory IL-10 production was also unlikely to be significantly different between these infections. While the actual relationship between superoxide production and ERK signaling is not yet known in our experimental system, it has been
previously shown that LPS-activated peritoneal macrophages induce Cu/Zn superoxide dismutase production through ERK activation \(^ {28}\). These results support previous studies indicating a need for peroxynitrite to control \(L.\ amazone\)nensis as a result of both superoxide and NO production \(^ {19}\). Although this relationship may also occur in macrophages infected with \(L.\ major\) amastigotes, we have demonstrated that the killing of \(L.\ major\) within activated macrophages is more directly related to the presence of nitric oxide and does not depend on the presence of superoxide (Fig. 4).

Although we did not see significant differences in either nitrite production or iNOS protein expression levels (Fig. 1 b and c) after macrophage activation, there were consistently higher numbers of \(L.\ amazone\)nensis amastigotes than \(L.\ major\) amastigotes within activated macrophages and an increased percentage of macrophages infected with \(L.\ amazone\)nensis. Recent studies have demonstrated that not only is iNOS generated NO important for leishmanicidal activity, but also that LOHA (an intermediate of iNOS dependent NO generation) can have a direct effect in inhibiting the growth of \(Leishmania\) \(^ {7}\). These previous studies demonstrated that both \(L.\ major\) and \(L.\ infantum\) were susceptible to the inhibitory effects of this compound at a concentration of 100 \(\mu M\) \(^ {7}\). We found that in contrast to \(L.\ major\), there was no growth inhibition of \(L.\ amazone\)nensis at a LOHA concentration of 500 \(\mu M\) (Fig. 2). Although the exact mechanism of this resistance remains unknown, there are at least three different possible mechanisms of LOHA resistance. First, parasite derived arginases could be resistant to LOHA inhibition. Recent studies have shown that arginase from \(L.\ amazone\)nensis was distinct from that of \(L.\ major\) and therefore these differences could provide \(L.\ amazone\)nensis-specific resistance to LOHA \(^ {29}\). Secondly, \(L.\ amazone\)nensis may
produce polyamines by other pathways, including arginine decarboxylase, shown to play a role in *Trypanosoma cruzi* metabolism. Finally, *L. amazonensis* may have the capability to use host-derived precursors to polyamine synthesis more efficiently than *L. major*. These three alternatives provide means by which *L. amazonensis* may use to counteract the inhibitory properties of LOHA.

Parasite replication has been previously demonstrated to correlate positively with the level of *L. major* infection using *in vitro* macrophage infection rates and our results with *L. major* amastigotes using both LOHA and hydroxyurea are consistent with these observations. Surprisingly, inhibition of parasite replication with hydroxyurea had only modest effects on limiting the infection rate of macrophages infected with *L. amazonensis* amastigotes (Fig. 2c). The absence of any further reduction in the numbers of parasites per macrophage in activated macrophages with hydroxyurea would suggest that there is no significant replication of *L. amazonensis* occurring during these activated conditions. Again this supports previous studies suggesting NO is cytostatic for *L. amazonensis*, but not cytotoxic. Therefore replication of *L. amazonensis* was not responsible for the resistance of this parasite to macrophage activation when compared to *L. major* (Fig. 2).

The studies presented here indicate that *L. amazonensis* amastigotes are resistant to host-mediated anti-leishmanial responses in non-inflammatory macrophages from C3H mice activated with the proinflammatory mediators LPS and IFN-γ. Furthermore, we demonstrate several significant differences in the host-parasite relationship when *L. amazonensis* amastigotes and *L. major* amastigotes are directly compared in our *in vitro* assay. We have found that although *L. amazonensis* is resistant to the growth inhibitory effects of LOHA the parasite does not require enhanced replication or iNOS inhibition for enhanced survival.
within the activated macrophages. While *L. major* amastigotes were killed primarily by NO alone, the anti-leishmanicidal response towards *L. amazonensis* amastigotes required both superoxide and NO production. Finally, these studies, implicate the ERK pathway as a host-signaling pathway potentially central to influencing the host-pathogen relationship of these intracellular parasites, warranting further study.

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Figure 1. *Leishmania amazonensis* amastigotes are resistant to killing by LPS/IFN-γ activated macrophages. A. C3HeB/FeJ BMM were infected with *L. amazonensis* or *L. major* amastigotes and activated 24 h later with IFNγ and LPS. At the timepoints post-activation indicated, the percent of the macrophages infected with parasites was determined. % of control = ((percent of infected macrophages in the activated cultures)/(percent of infected macrophages in the non-activated culture))x100. The data shown are the mean + the standard error from 3 independent experiments with 2 replicates per experiment. (*= p<0.05, Scheffe's test) B. Nitrite levels in supernatants of the cultures measured by the Greiss reaction. Results
are the average of 3 experiments with 2 to 3 replicates in each experiment. C. The level of iNOS protein in the cytoplasmic extracts of uninfected (U), *L. amazonensis* (LA), or *L. major* (LM) infected macrophages, was determined at 24 hours after activation via western blot analysis with an anti-iNOS antibody and re-hybridized to anti-actin. The results are representative of 3 independent experiments. D. The number of parasites per infected macrophage was determined 3 days after activation. The data shown are the mean + standard error from 3 independent experiments with 2 to 3 replicates per experiment. (*= p<0.05, paired t-test compared to control).
Figure 2. *Leishmania amazonensis* amastigotes are resistant to the arginase inhibitor N° Hydroxy-nor-L-arginine (LOHA). C3HeB/FeJ BMM were infected and at 24 hours post-infection left non-activated or activated as described in Fig. 1 and assayed for (A and C) the percent infected macrophages or (B and D) the number of parasites per macrophage. 500 μM LOHA (Gray bars) or 4mM hydroxyurea (black bars) was added to the appropriate wells at 24 hours post-infection. Both figures A and B were under non-activation condition while figures C and D were activated with LPS and IFNγ. The data shown are for day 3 after treatment and activation (*= p<0.05, paired t-test). The results shown are the mean ± standard error of 3 independent experiments with 2 replicates per experiment.
Figure 3. *L. amazonensis* amastigotes are resistant to NO within infected macrophages. C3HeB/FeJ BMM were infected as described in Fig. 1. At 24 hours post-infection (a) cells were either left non-activated with 100 μM SNAP (SNAP alone) or activated with IFN-γ/LPS as in figure 1 (Activated) or activated with SNAP (Activated + SNAP). (b) cells were activated without (Activated) or with 1 mM 1-NIL (Activated + 1-NIL). The percentage of macrophages infected with parasites was determined after 3 days as described in figure 1. The data shown are the mean ± standard error from 2-3 independent experiments with 2 replicates in each experiment. * indicates significant differences (p< 0.05, paired t-test).
Figure 4. The ERK inhibitor PD98059 promotes superoxide and iNOS dependent killing of *L. amazonensis* amastigotes. C3HeB/FeJ BMM were infected and activated with LPS/IFN-γ as described in figure 1. At the time of activation the ERK inhibitor, PD98059 (20 mM), the cell permeable superoxide dismutase mimetic, MnTBAP (15 mM), or the iNOS inhibitor, 1-NIL (1 mM), was added as indicated. The data shown are the mean ± standard error from 2-3 independent experiments with 2 replicates in each experiment (* indicates a significant difference (p< 0.05, paired t-test), the letter (a) indicates significant difference (p< 0.05, paired t-test) with *L. amazonensis* infected BMM activated and treated with PD98059 alone and the letter (b) indicates significant difference (p< 0.05, paired t-test) with *L. major* infected BMM activated and treated with PD98059 alone.
CHAPTER 3

Leishmania major specific CD4$^+$ T cells and B cells limit L. amazonensis amastigote survival within in vitro infected macrophages through IgG mediated superoxide production

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Abstract

Infection of C3H mice with L. amazonensis results in a persistent lesion characterized by an immune effector T cell phenotype that is neither a Th1 or Th2 response, while infection with L. major results in a Th1 response and a healed lesion by 8-10 weeks post-infection. Previous work from our lab has shown that a prior infection with L. major will protect against a subsequent challenge of L. amazonensis. We now show that draining lymph node cells (DLN) of L. major infected mice are able to limit both the percentage of macrophages infected with L. amazonensis amastigotes in vitro and the number of parasites per macrophage in an antigen-dependent manner. Depleting either CD4$^+$ T cells or B cells abrogated the ability of the DLN cell population to control the infection, whereas depleting CD8$^+$ or CD11b$^+$ cells had a minimal effect. CD4$^+$ T and B cells together were sufficient to
induce effective macrophage activation although direct contact between the lymph node cells and the macrophages was not necessary. Limiting secreted antibodies from the culture using anti-IgG/IgM coated beads significantly inhibited killing of the parasite in this experimental system. Inhibiting intracellular superoxide production with MnTBAP treatment caused a significant increase in *L. amazonensis* survival. In conclusion, ex-vivo derived CD4\(^+\) T cells and B cells from the DLN of *L. major* infected mice can activate macrophages to control *L. amazonensis* infection through antibody-mediated superoxide production.

**Introduction**

Infecting C3H mice with *L. amazonensis* results in a persistent, high parasitic load, and chronic lesion. Infection of these same mice with *L. major* results in lesion size peaking within 2-3 weeks, which is completely healed 8-10 weeks later. These differences in clinical presentation relate directly to the effectiveness of the immune response towards parasites and the characterization of the deficiency in the immune response to *L. amazonensis* and the mechanism to evade the immune system are the focus of many studies up to this point. In vivo, *Leishmania* amastigotes establish their infection within host macrophages and their survival is dependent in part on their resistance to macrophage-mediated killing. This resistance could be through direct effects of the parasite on the macrophage itself to inhibit activation or through their effect on other immune cell types that participate in the induction of macrophage activation and killing.

Several studies have established a role for multiple cell types of the immune system in the immune response to *Leishmania* using either genetically altered mice or antibodies targeting a specific cell type in vivo (reviewed (1)). CD4\(^+\) T cells were identified as a key
cell type in controlling *Leishmania major* infection. Specifically, whether they induce healing and parasite control through a Th1 response with high IL-12 and IFN-γ and or a non-healing Th2 response with high IL-10 and IL-4, also determined by the antigen specific CD4⁺ T cell response (reviewed (2)). However, the collective data up to now indicates that the production of a protective immune response toward *L. amazonensis* is not as simple as the presence of a Th1 response. Vaccine designs using DNA or promastigotes gave some protection against *L. amazonensis* infection (3, 4, 5, 6) but an antigen specific Th1 response induced by either transferring a *L. amazonensis* Th1 cell line to C57BL/6 mice (7) or injecting IL-12 and dendritic cells pulsed with *L. amazonensis* antigen to lesions of C3H mice infected with *L. amazonensis* (8), did not resolve the infection. However, a primary infection with *L. major* infection does induce a healing response after *L. amazonensis* challenge (9). Together these studies indicate that CD4⁺ Th1 cells alone may not be able to control *L. amazonensis* infection although the immune response developed during a healing *L. major* infection can provide all necessary factors to resolve a secondary *L. amazonensis* infection.

Contradictory reports exist for the role of B cells, antibody and antibody receptors in *Leishmania* infection. Most studies show lack of necessity for B cells and/or antibodies in healing and indicate a role of antibodies and Fc receptors in *Leishmania* cellular invasion (10). It has been also shown that internalization of antibody opsonized *Leishmania* induces IL-10, inhibiting macrophage activation (11, 12, 13). Separate studies have demonstrated that activation of macrophages by IgG through Fc receptors in the presence of IFNγ results in superoxide and hydrogen peroxide production, as well as nitric oxide production (14).
As we have found in previous studies that infection with *L. major* can limit a subsequent challenge with *L. amazonensis*, we tested the hypothesis that DLN cells from mice infected with *L. major* could control *L. amazonensis* amastigote infection of BMM in vitro. We found that CD4+ T cells and B cells from DLN of *L. major* infected mice were, together, in the presence of antigen capable of inducing BMM to control *L. amazonensis* amastigotes infection. Specifically, secreted factors from these cells were found to be responsible for the effective BMM activation. One secreted factor identified was immunoglobulin and the mechanism of parasite killing was superoxide dependent. These findings provide crucial informative knowledge to provide effective improved treatments and vaccination strategies against particular *Leishmania* spp. and potentially other intracellular pathogen that cause chronic disease by indicating the importance of inducing a protective antibody response.

2. Material and Methods:

1.1 2.1 Mice

C3HeB/FeJ (C3H) and C3Smn.CB17-Prkda<sup>scid</sup>/J (C3H scid) mice were obtained from Jackson Laboratory (Bar Harbor, Maine). The mice were maintained in a specific-pathogen-free facility. Mice were infected with 5x10⁶ stationary-phase promastigotes in 50 μl of PBS in the left hind footpad. All procedures involving animals were approved by the Committee on Animal Care at Iowa State University.
2.2 Parasites

*Leishmania major* (MHOM/IL/80/Freidlin) and *Leishmania amazonensis* (MHOM/BR/00/LTB0016) promastigotes were grown to stationary phase in Grace’s insect culture medium (Technologies, Gaithersburg, MD) with 20% heat-inactivated FCS, 2 mM glutamine, 100 U penicillin per ml, and 100 μg of streptomycin per ml. Amastigotes used for infection were tissue derived and were harvested from lesions of C3H SCID mice as previously described (15). Freeze-thawed *Leishmania* antigen (Ag) was obtained from stationary-phase promastigotes as described (16).

2.3 Cells and cell culture

Cells were obtained from bone marrow (15-20 x 10⁶ cells) and plated in a 150 x 15 mm Petri dish with 30 ml of macrophage medium containing 30% L-cell conditioned medium, 20% FCS and 50% Dulbecco’s modification of eagle’s medium (DMEM), 2 mM glutamine, 100 U penicillin per ml, 100 μg of streptomycin per ml, and 1 mM sodium pyruvate at 37 °C and 5% CO₂. After 2 days another 20 ml of macrophage medium was added. At day 6 the non-adherent cells were washed and adherent cell populations were harvested by placing the plates on ice for 20 min and then scraping with a tissue scraper. After washing in PBS, the live cells were counted using trypan blue exclusion and resuspended in complete tissue culture medium (CTCM) containing DMEM, 10% FCS, 2 mM glutamine, 100 U penicillin per ml, 100 μg of streptomycin per ml, 25 mM 4(2-Hydroxyethyl)piperazine-1-ethane sulfonic acid (HEPES), and 0.05 μM 2-β-mercaptoethanol.
Lymph node cells were obtained from the LN draining infection site (left hind foot) of animals infected for 4 weeks with either *L. amazonensis* or *L. major*. The poplitial, inguinal and prescapular LN were collected from non-infected mice. LN from 4-10 mice were pooled in 2 ml CTCM and the cells separated with a 2 ml tissue grinder, then washed with 10 ml CTCM at 300xg, 4 °C for 10 min. LN cells were re-suspended in 4 ml CTCM and passed through 40 μm nylon cell strainer (BD Falcon, Bedford, MA). Lymph nodes cells were counted using trypan blue exclusion.

2.4 Lymph node cell sorting

CD4⁺ T cells, B cells, CD8⁺ T cells, MHCII⁺ cells, and CD11b⁺ were depleted by incubating the cells first for 5 min at room temperature with one of the following primary antibodies per 1x10⁸ lymph node cells; Rat anti-mouse CD4 (15μg), CD19 (25μg), CD8α (10μg), MHCII (I-A^k, 25 μg) or CD11b (12 μg) (LEAF, Biolegend, San Diego, CA), then washed. Cells were incubated for 15 min at 4 °C with the goat anti-rat IgG Microbeads (Miltenyi Biotec, Auburn, CA) according to manufacturer directions. A kit for negative selection of CD4⁺ T cells (Miltenyi Biotec, Auburn, CA) and B cells (MagCellect, R&D system, Minneapolis, MN) were used as directed by manufacturers. Cells were then separated using an autoMACS™ separator (Miltenyi Biotec, Auburn, CA). After washing the sorted cells with PBS once, they were re-suspended in 1 ml CTCM and counted using trypan blue exclusion. Cell purity was assessed using FACS analysis (see Fig A 4, 5 and 6).
2.5 Macrophage infection and cell co-culture

BMM were plated in 24 well plates with glass coverslips at a rate of $5 \times 10^3$ cells/well, in 1 ml CTCM. After 24 h the BMM were infected with either *L. major* or *L. amazonensis* amastigotes at a 3:1 ratio and incubated at 34°C for 24 h. The wells were washed two times with DMEM to remove extracellular amastigotes and 500 \( \mu l \) of CTCM was added. Lymph node cells were added with Ag (50 \( \mu g/ml \)) and the volume was adjusted to 1 ml CTCM. 10 \( \mu M \) Mn(III)Tetrakis(4-benzoic acid) porphyrin chloride (MnTBAP, BIOMOL, Plymouth Meeting, PA) were added at the time of LN cell addition as indicated. The coverslips were removed after 5 days incubation at 34 °C with 5% CO \(_2\), and stained using nonspecific HEMA 3 stain set (Fisher Diagnostics, Middletown, VA) and mounted on glass slides.

2.6 Determination of the infection rate of macrophages and parasite count

Coverslips were counted under the light microscope by examining 3 areas at 1000x total magnification. In each area, 100 macrophages were examined and the number of infected macrophages and the number of parasites in each macrophage were counted. The average of the 3 areas was used to determine the percent of infected macrophages and the number parasites per macrophage on each coverslip.

2.7 Antibody Coating beads

Polystyrene beads (Interfacial Dynamic Co., Tualatin, OR) were washed 2 times with 2 ml sterile PBS in 5 ml tube and centrifuged at 453g for 10 min at 4°C. The beads were
then counted using a hemocytometer and resuspended in 500 μl PBS. A specific number of beads was incubated with the antibodies; goat anti-mouse IgG/IgM or goat anti-rat IgG (Jackson ImmunoResearch Laboratories, West Grove, Pa) at a concentration of 12.5 μg/ml at 4 °C with shaking, overnight. Beads were washed 2 times with CTCM and centrifuged at 453xg for 10 min at 4 °C then resuspended in 1 ml CTCM and incubated at room temperature for 1 h. Beads were stored at 4 °C until use. Beads were added at 15x10^6 beads/well.

2.8 Western blot

The supernates of the cultures either without beads, with goat anti-mouse IgG/IgM or goat anti-rat IgG coated beads were used to measure antibody levels. 300 μl of the supernatant from these cultures was mixed with 30 μl Protein A Agarose beads and incubated overnight at 4 °C on the shaker. The tubes were centrifuged at 13000xg for 1 min, the supernatant was removed, the beads were washed once with PBS, and resuspended in 25 μl lysis buffer (0.32 M sucrose, 3 mM calcium chloride, 2 mM magnesium acetate, 0.1 mM EDTA, 10 mM Tris(Hydroxyethyl) aminomethane (Tris) (pH 8.0), 5 mM sodium fluoride, 1 mM dithiothritol (DTT), 0.5 mM phenylmethanesulfonyl fluoride (PMSF), 0.1 mM sodium orthovanadate and 1% nonidet P-40). 5 μl of 6x loading buffer was added and the mixture was heated at 95 °C for 4 min. Samples were separated using 8% PAGE and transferred to a nitrocellulose membrane using a semi-dry blotting apparatus (Bio-Rad, Hercules, CA). The membrane was blocked with 5% nonfat dry milk in PBS 0.1% Tween. Peroxidase-conjugated goat anti-mouse IgG (H+L) (Jackson ImmunoResearch, West Grove, PA) was
hybridized to the membrane overnight at 4 °C at a 1:25000 dilution. Bound peroxidase conjugated antibody was detected using Pierce Supersignal Reagents (Pierce, Rockford, IL) as directed by the manufacturer.

2.9 Determination of nitric oxide and IFN-γ

Nitrite concentrations were determined using the Greiss reagent as described previously (17). Briefly, equal volumes (50 µl) of cell culture medium and Greiss reagent (LabChem, Pittsburgh, PA) were mixed and incubated at room temperature and the absorbance was measured at 550 nm with a microplate reader (Molecular Devices, Sunnyvale, CA). The nitrite concentration was determined using a standard curve generated with sodium nitrite. Levels of IFN-γ protein were determined by ELISA with commercially available antibodies (Pharmingen, San Diego, Ca), peroxidase-conjugated streptavidin (Jackson ImmunoResearch Laboratories, West Grove, Pa.) and ABTS microwell peroxidase substrate (Kirkegaard & Perry Laboratories, Gaithersburg, Md.) according to manufacturer recommendations.

2.10 Statistical analysis

Data analysis was performed by using Statview 5.0.1 (SAS, Cary, NC). Statistical significance (set at p<0.05) was determined paired using t-test as indicated.
Results

Total lymph node cells from mice infected with L. major can induce BMM to kill L. amazonensis amastigotes in vitro via L. major specific antigen.

Previous in vivo studies from our laboratory show that re-challenging healed L. major infected mice with L. amazonensis 11-15 weeks post-infection leads to resolution of their L. amazonensis infection (9). Here we test the hypothesis that LN cells draining the infection site of L. major infected mice can activate in vitro BMM infected with L. amazonensis to control infection. Mice were infected in the footpad with either L. amazonensis or L. major promastigotes for 4 weeks, the time by which the lesion in mice infected with L. major was starting to heal. The draining LNs were removed, pooled and disaggregated into single cell suspension. LN cells were placed in culture with BMM macrophages previously infected for 24 hrs with either L. amazonensis or L. major amastigotes. The co-cultures were then incubated with parasite Ag corresponding to the primary in vivo infection, for example LN cells derived from L. major infected mice were stimulated with L. major antigen and incubated for 5 days at 34 °C. Total lymph nodes cells from L. major infected mice caused a significant reduction in both the percent of macrophages infected and the number of parasites per macrophage in L. amazonensis infected BMM compared to LN cells from L. amazonensis infected mice (Fig. 1a). This reduction was antigen dependent (data not shown and Fig. 2). As seen in Figure1c, vacuoles in the L. amazonensis infected macrophages were smaller and fewer parasites per macrophage were seen after adding LN cells from L. major infected mice compared to adding LN cells from L. amazonensis infected mice (Fig. 1b). These findings indicate that stimulated immune cells from L. major infection can induce BMM infected with L. amazonensis to reduce an in vitro infection.
*L. amazonensis* Ag failed to induce *L. major* LN cells to activate BMM to reduce *L. amazonensis* infection, reduction in infection rate is the reduction in the percent infected BMM compared to percent infected BMM after the addition of LN cells from *L. amazonensis* mice (Fig 2a), implicating a specific requirement for *L. major* Ag to stimulate *L. major* lymph node cells and produce killing in *L. amazonensis* infected BMM. However, after *L. amazonensis* Ag was added, *L. major* LN cells induced a reduction in the percent of *L. major* infected macrophages (Fig 2b), indicating that *L. amazonensis* Ag does not inhibit either BMM activation or DLN cells response.

To confirm this specificity of *L. major* LN cells for *L. major* Ag, LN cells from non-infected (C3H) mice were co-cultured with *L. amazonensis* infected BMM and to either *L. amazonensis* Ag, *L. major* Ag or no Ag (Fig 2c). These naïve LN cells were not able to induce activation of macrophages regardless of Ag source (Fig. 2c). Therefore, the response that controls *L. amazonensis* infection in BMM is specific to DLN cells derived from *L. major* infected mice and restimulation with *L. major* Ag.

**CD4+ T cells are necessary, but not sufficient to induce BMM to control L. amazonensis infection.**

The population of total lymph node cells includes multiple cell types that may participate in macrophage activation. The importance of CD4+ T cells in vivo in controlling *Leishmania* infection has been well characterized (reviewed in (18)). To test whether CD4+ T cells are necessary and sufficient alone to induce BMM activation in our system, we either depleted CD4+ T cells from the DLN cell population by positive selection or negatively selected CD4+ T cells to use in the in vitro assay. The results in figure 3a clearly demonstrate
that CD4\(^+\) T cells are necessary for BMM activation and *L. amazonensis* killing. In contrast, co-culture of CD4\(^+\) T cells alone with *L. amazonensis* infected macrophages was not sufficient to induce killing of the parasite (Fig. 3a). These results suggested other cells, in addition to CD4\(^+\) T cells, are necessary to reduce BMM infection. Other cell types thought to participate in controlling intracellular parasite infection include; CD8\(^+\) T cells, CD11b\(^+\) (macrophages and dendritic cells) and/or MHCII positive cells (macrophages, DC, and B cells). Depletion of CD8\(^+\) T cells did not have a significant effect on ability of macrophages to the kill *L. amazonensis* parasites while limited significant effect was seen with CD11b\(^+\) cells depletion (Fig 3b). The combination of CD4\(^+\) T cells and CD8\(^+\)T cells also did not induce macrophages to induce reduction in *L. amazonensis* infection (data not shown). However, depletion of MHCII positive cells did significantly reduce parasite killing with almost completely abrogating the effectiveness of the DLN cells (Fig. 3b). Therefore, a long with CD4\(^+\) T cells, a population of MHCII/CD11b\(^+\) cells are critical to effectively activate *L. amazonensis* infected macrophages to kill intracellular *L. amazonensis*.

*B cells and CD4\(^+\) T from draining lymph nodes of L. major infected mice, together, are sufficient to control L. amazonensis infection.*

The MHCII/CD11b\(^+\) cell type of highest abundance are B cells. To test whether B cells play a role in macrophage activation to kill intracellular *L. amazonensis*, the DLN cells were depleted of B cells and then co-cultured with *L. amazonensis* infected BMM as previously described. Removal of B cells by positive selection using an anti-mouse CD19 antibody abrogated the ability of DLN cells to activate macrophages to kill *L. amazonensis* parasites (Fig. 4a). Addition of negatively selected B cells, tested as CD19\(^+\) cells, as
described in the material and methods to *L. amazonensis* infected macrophages was not sufficient to control infection (Fig. 4a). However, combining B cells and CD4+ T cells isolated from draining lymph nodes cells of *L. major* infected mice was sufficient to activate macrophages and recover the majority of parasite killing characteristic of the total DLN cell population (Fig 4a). Mixing B cells from *L. major* infected mice with CD4+ T cells from *L. amazonensis* infected mice or mixing CD4+ T cells from *L. major* infected mice with B cells from *L. amazonensis* infected mice did not induce a reduction in *L. amazonensis* infection (Fig. 4b). These results indicates that both the CD4+ T cells and B cells must be from mice infected with *L. major* (Fig. 4b), suggesting that both CD4+ T cell and B cells from *L. amazonensis* infected mice are deficient in critical effectors molecules which stimulate infected macrophages to kill *L. amazonensis* parasites.

*Soluble factors are responsible for controlling L. amazonensis infection in BMM.*

To define the mechanism by which lymph nodes cells activate infected macrophages, we studied whether direct contact between the LN cells and macrophages is necessary. Therefore we used total DLN cells or purified CD4+ T cells and B cells in a transwell system as described in the material and methods. We found that effective macrophage activation was not dependent upon direct contact (Fig 5a), although CD4+ T cells did require interaction with a MHCII+ cells via direct contact with either the macrophage or B cells (Fig. 5a).

The requirement for a soluble factor from B cells suggested a role for antibody in this in vitro system. To test this, goat anti-mouse IgG-IgM coated polystyrene beads were used in the transwell system with total LN cells draining *L. major* infected mice and goat anti-rat IgG coated beads were used as a control. Inclusion of the anti-mouse IgG/TgM coated beads
significantly reduced the ability of DLN cells to promote killing of intracellular *L. amazonensis* amastigotes (Fig 5b). Antibody reduction did not play a significant role in the control of *L. major* infection in BMM (Fig. 5b). Supernates from these cultures were analyzed via western blot with anti-mouse IgG to confirm a reduction of IgG in bead added cultures. IgG levels were significantly reduced in supernates of co-culture with anti-IgG coated beads as compared to the supernates of total LN cells, without beads (Fig 5c and d). These studies show that control of *L. amazonensis* infection, but not *L. major* infection, by *L. major* LN cells in the infected BMM co-culture assay is antibody dependent.

*Lymph node cells from L. major infected mice induce the control of L. amazonensis infection through a superoxide dependent pathway.*

IFN-γ is the main Th1 cytokine produced from CD4+ T cells and a potent macrophage activator. However, no correlation was found between the ability of the macrophage to induce parasite killing and IFN-γ production in the co-culture system ($R^2 = 0.045$) (Fig 6a). Nitrite levels were also measured in the supernates, as a reflection of nitric oxide levels which is a common measure of macrophage activation. No direct correlation was found between the infection rate and nitrite levels ($R^2 = 0.0046$) (Fig 6b). These results do not exclude the importance of IFN-γ and NO in this system, but it suggests that there are other factors in our in vitro system which may significantly enhance the ability of activated macrophages to kill *L. amazonensis* amastigotes.

A previous study using liposomal-IgG to ligate Fc receptors on *L. donovani* infected macrophages, showed that this ligation initiated control of infection through superoxide and
hydrogen peroxide production (14). We hypothesized that antibodies produced by B cells within the population of LN cells from *L. major*-infected mice may stimulate macrophages through a superoxide dependent pathway to promote parasite killing. Addition of a cell permeable superoxide dismutase mimic (MnTBAP) to cultures abrogated the killing almost completely, while although the *L. major* infection rate was affected, it was inhibited to a much lesser extent (35.01 to 20.62%) (Fig. 6c). Killing of *L. amazonensis* is therefore dependent on superoxide production. In conclusion, these results suggest that CD4+ T cells and B cells from DLN cells of *L. major* infected mice induce intracellular *L. amazonensis* killing, and secreted factors including antibodies induce this killing through a superoxide dependent mechanism.

Discussion

This work reports for the first time the ability of draining lymph node cells from *L. major* infected C3H mice to activate BMM and reduce the *L. amazonensis* amastigotes infection rate in vitro (Fig. 1). Also proposes a mechanism by which this reduction occurs. These findings are supported by previous in vivo data from our lab in which healed *L. major* infected mice were able to cure a secondary *L. amazonensis* infection (9). The immune response from *L. major* infected mice that induced *L. amazonensis* killing is specific for *L. major* Ag and not *L. amazonensis* Ag (Fig. 2a). *L. major* antigen alone could not induce activation of lymph nodes cells obtained from naïve or *L. amazonensis* infected mice (Fig 2 b and c), indicating it is not the activity of the *L. major* Ag, but the specificity of the immune response and lymphocytes to *L. major* Ag that induces the activation in the macrophages.
Previous in vivo studies have shown the importance of CD4\(^+\) T cells in promoting healing of *Leishmania* infection (reviewed (18)). The necessity for CD4\(^+\) T cells in our co-culture system (Fig. 2) is consistent with this understanding of the anti-*Leishmania* immune response. Despite this, it has been previously reported that removal of CD4\(^+\) T cells leads to diminished pathology and decreased parasite counts after *L. amazonensis* infection in C57BL/6 mice, indicating a role for CD4\(^+\) T cells in the pathogenesis of this parasite (19). In our system, CD4\(^+\) T cells are necessary for the reduction in the infection rate, but they are alone not sufficient to induce this reduction (Fig. 3a). Unexpectedly, the additional required cell type was not CD8\(^+\) T cells or dendritic cells (Fig 3b). To date, the literature varies in its determination for the role of CD8\(^+\) T cells in clearing *Leishmania* infection, results differ based on the parasite dose or species and vaccine strategy (20, 21, 22). A role for CD8\(^+\) T cells in producing IFN-\(\gamma\) to augment CD4\(^+\) T cell responses also has been shown (23, 24). In our system, CD8\(^+\) T cells were not found to be necessary (Fig. 3b) and neither dendritic cells (DCs) nor macrophages, both of which express CD11b, play a major role in our system (Fig3b). DCs do have a role in initiating the immune response and in presenting Ag to CD4\(^+\) T cells (25).

The role of B cells in an effective immune response to *Leishmania* in murine model is controversial and may be dependent on the *Leishmania* species and/or strain of mice (reviewed in (1)). Many previous reports showed that either B cells are not necessary (26, 27) or even that the immune response was enhanced in controlling infection in the absence of B cells (28, 29). Only a single study done using C3H mice infected with *L. major* shows the necessity for B cells to generate a healing T cell response (30). In our experimental system, B
cells were found to be necessary but not sufficient alone to activate macrophages, but together with CD4\(^+\) T cells could activate the macrophages to kill *L. amazonensis* (Fig. 4a).

The inability to substitute either CD4\(^+\) T cells or B cells with the same cell types isolated from LN of *L. amazonensis* infected mice (Fig 4b) might suggest not only a defect in CD4\(^+\) T cells from *L. amazonensis* infected mice described previously (8, 16, 31, 19), but also a defect in B cells from *L. amazonensis* infected mice, particularly in their ability to secret necessary factor(s) to induce the proper BMM activation for parasite killing. Part of this defect could be as a result of the poor CD4\(^+\) T cells response, which in turn may not induce an effective B cell response. Lower levels of IgG2a has been shown in the serum of *L. amazonensis* infected mice in vivo as compared to the serum from *L. major* infected mice (16). In our system, lower levels of total IgG and levels of IgG2a were detected in the culture of LN cells from *L. amazonensis* infected mice compared to LN cells from *L. major* infected mice (Figure A 3). This is not only an indication of a poor Th1 response, but may be also reflect a defect in B cells is induced by *L. amazonensis* infection.

Lemos, (32) showed macrophage MHCII/CD4\(^+\) T cells contact is not necessary for macrophage activation. In figure 4a, a transwell system ruled out the necessity of direct contact for BMM activation in our co-culture system. This result does not exclude the need for contact between CD4\(^+\) T cells and MHCII bearing cells in vivo to promote T cell activation. Instead it signifies that in this system B cells are not acting only as APC for CD4\(^+\) T cells, but instead suggests that secreted factor(s) produced from B cells participate in the control of *L. amazonensis* infection. These findings indicate that there are critical elements produced by B cells which promote macrophage killing of these intracellular parasite.
The role of antibodies in *Leishmania* infection is as controversial as the role of B cells. Antibodies have been shown to play a role in the resistance of opsonized parasite to macrophage activation by inducing IL-10 production (11). Antibody is also crucial to *L. mexicana* entry into macrophages via the Fc receptors binding and sustenance of the infection (10). Conversely, antibodies were reported to induce the control of *Leishmania* infection in vitro (14) and in vivo (33). In our system the antibodies were necessary to induce macrophages to kill *L. amazonensis*. After addition of antibody depleting beads to the co-culture, a low level of antibody was still detected (Fig 5d). The incomplete abrogation of killing could be due to this remnant level of antibodies in the culture (Fig 5b).

Recently, we have shown NO production was sufficient for BMM infected with *L. major* amastigotes to control infection, while control of *L. amazonensis* infection requires both NO and superoxide production (Mukbel, et al., 2005, submitted). Control of *L. major* infection in our in vitro assay was not affected by a reduction of antibody levels in the culture (Fig 5b), supporting our previous finding that superoxide is not required to control *L. major* infection. Inhibition of superoxide using MnTBAP induced a minimal effect in parasite reduction during *L. major* infection, while a dramatic abrogation of *L. amazonensis* killing was seen (Fig 6c). No correlation was found with either IFNγ or NO and the ability of macrophages to control *L. amazonensis* infection (Fig 5a and b). IFNγ itself was shown to enhance *L. amazonensis* replication (7) which might explain the absent of direct correlation between IFN-γ and killing of *L. amazonensis* amastigotes.

In conclusion both B cells and CD4+ T cells from DLN cells of *L. major* infected mice are necessary to activate macrophages to kill intracellular *L. amazonensis* amastigotes.
within. This response is antigen dependent and requires secreted factors produced from the two cell types including antibodies from B cells. These secreted factors induced L. amazonensis killing in a manner utilizing superoxide production. This link, indicated by the involvement of both CD4⁺ T cells and B cells in our system, between humoral and cell-mediated immunity may be critical for vaccine development against Leishmania.

References


Figure 1. DLN cells isolated from mice infected for 4 week with *L. major* induce BMM to reduce *L. amazonensis* infection as compared to DLN cells isolated from *L. amazonensis* infected mice. C3H mice were infected with $5 \times 10^6$ *L. major* or *L. amazonensis* promastigotes in the left footpad. 4 weeks post-infection the DLN were removed and $5 \times 10^8$ total cells were added on top of $5 \times 10^5$ BMM previously infected with $1.5 \times 10^6$ *L. amazonensis* amastigotes as described in material and methods. Antigen (50µg/ml), *L. amazonensis* Ag or *L. major* Ag to cultures with LN cells from mice infected with *L. amazonensis* or *L. major*, respectively, was added and the cells were incubated for 5 days at 34 °C. A) Percent infected macrophages and
number of parasites per infected macrophage was calculated as described in the materials and methods. The results are the average of 8 experiments ± standard error. Figure b) Image of BMM macrophages infected with *L. amazonensis* after the addition of LN cells from *L. amazonensis* infected mice. C) Macrophages the same as b with LN cells from *L. major* infected mice.
Figure 2. Both DLN cells from *L. major* infected mice and *L. major* derived antigen are needed to induce a reduction in infection rate of *L. amazonensis* infected mice. LN cells were obtained as described in figure 1. a) *L. amazonensis* infected BMM were co-incubated with LN cells from *L. major* infected mice (*L. major LN*) and either *L. major* or *L. amazonensis*
antigen (*L. major* Ag or *L. amaz.* Ag), or *L. amazonensis* LN cells (*L. amaz.* Ag) with *L. major* antigen. Reduction in % infected macrophages = (1 - (% infected macrophages with LN cells from *L. major* infected mice / % infected macrophages with LN from *L. amazonensis* infected mice)) * 100.  

b) *L. major* infected macrophages were incubated with LN cells from *L. major* infected mice and either *L. major* antigen or *L. amazonensis* antigen.  

c) *L. amazonensis* infected BMM were incubated with LN node cells from non-infected mice with no antigen, *L. amazonensis* antigen, or *L. major* antigen. Results are an average of 3 experiments ± S.E. (*) infection rate is higher than the control i.e. infection rate of experimental groups was higher than the infection rate of *L. amazonensis* infected macrophage with LN cells from *L. amazonensis* infected mice).
Figure 3. Both CD4⁺ T cells and MHCII⁺/CD11b⁻ cell types are necessary to induce BMM to reduce *L. amazonensis* infection rate. DLN cells of *L. major* infected mice, were harvested as described in the figure 1. a) CD4⁺ T cells were depleted from DLN cell population by positive selection or CD4⁺ T cells were enriched by negative selection as described in the material and methods and used in the co-culture with *L. amazonensis* infected BMM as described in figure 1. The results are average of 5 experiments ±S.E. b). MHCII, CD8 or CD11b positive cells were depleted from the DLN cells of *L. major* infected mice as described in the materials and methods and co-cultured with *L. amazonensis* BMM as in fig 3a. The results are average of 3 experiments with ± S.E.

*= Statistically significant from the Total DLN cells
Figure 4. *L. major*-specific B cells and CD4+ T cells together are necessary to control *L. amazonensis* infection in vitro. LN cells from *L. major* infected mice were isolated as described in Figure 1. BMM infected with *L. amazonensis* amastigotes as in figure 1. a. Total LN cells (Total), B cell depleted (CD19+ cell depletion), B cells alone (B cell enrichment), or B cells and CD4+ T cells (B cells and CD4) were isolated as described in the material and methods. The results are the average of 3-4 experiments ±S.E. b. B cells and CD4+ T cells were sorted from LN cells of mice infected either with *L. amazonensis* or *L. major* (B cells *L. amazo.* or *L. major*; CD4+ T cells *L. amazo.* or *L. major*) and added to *L. amazonensis* infected BMM. Results represent an average of 2-3 experiments ±S.E. (*) infection rate was actually higher than the control as described in figure 2.
Figure 5. Secreted factors from B cells and CD4+ T cells are sufficient to reduce the infection rate of *L. amazonensis* infected BMM. DLN cells from *L. major* infected mice were used sorted and added as described in figure 4. a) Either total LN cells (Total LN) or negatively
sorted B cells and CD4⁺ T cells were added to the upper chamber or lower chamber (lower) as indicated in transwell with *L. amazonensis* infected BMM in the lower chamber, killing was determined as in figure 1. Results are the mean ± of 3 independent experiments. b) Total LN cells from *L. major* infected mice were added into the upper chamber of transwell as in a. either alone or with goat anti-mouse IgG/IgM coated beads to macrophages infected with either *L. amazonensis* or *L. major* as indicated. c) A representative image of an immuno-blot using goat anti-mouse IgG Protein A precipitated supernates of co-cultures separated with PAGE as described in the material and methods to detect levels of IgG in the transwell with or without the beads. d) An average of densitometry results of three experiments ±S.E., (*) p<0.05.
Figure 6. Reduction in the infection rate of BMM infected with *L. amazonensis* is superoxide dependent. The supernates of the cell cultures of *L. amazonensis* infected BMM with DLN cells types were used to quantified the amount of (a) IFNγ and (b) nitrite and plotted against infection rate of the co-infection. (c). total LN cells from *L. major* infected mice were obtained as described in figure 1 and added to BMM infected with either *L.amazonensis* or *L.*
major amastigotes, with or without 10 μM MnTBAP and incubated in the presence of antigen for 5 days. These results are the average of 3 experiments ± S.E. in duplicate. (*) p<0.05 compared to no MnTPAB treatment.
CHAPTER 4

Leishmania infection modulates the expression of Fcγ receptors

Paper prepared to be submitted to Infection and Immunity

Rami M. Mukbel and Douglas E. Jones

Abstract:

Antibody and Fc receptors were recently been shown to act as virulence factors for Leishmania infection susceptible mice. These host molecules are co-opted by the parasite to play a role in pathogenesis of Leishmania infection either as a mode of entry into the cell, triggering IL-10 production, or both. Recently, we demonstrated an antibody-mediated mechanism of macrophage activation utilized by draining lymph node cells isolated from Leishmania major infected mice which promotes killing of intracellular L. amazonensis amastigotes. In this previous work it was demonstrated that soluble factors from B cell and CD4+ T cells isolated from lymph node (LN) cells of L. major infected animals were able to promote macrophage killing of L. amazonensis amastigotes, that parasite killing was antibody mediated. In this work, using immune serum from mice chronically infected with L. major, we could not promote parasite killing. To further explore these findings, we wanted to measure FcγR expression on infected macrophages to test the hypothesis that these activation receptors may be differentially regulated by infection with L. amazonensis vs L. major. 24 hour post-infection with either L. major or L. amazonensis amastigotes, CD64 (FcγRI) and CD32 (FcγRII) expression was measured using RT-PCR. Both parasites induced down-
regulation of CD64 and up-regulation of CD32 expression in infected BMM. While addition of LN cells from either *L. amazonensis* infected mice or *L. major* infected mice had similar effects on macrophages CD64 expression, LN cells from *L. major* infected mice induced a downregulation of CD32 expression on BMM compared to LN cells from *L. amazonensis* infected mice. In conclusion, this is the first report to show that both infection with *Leishmania* spp. and addition of LN node cells from *Leishmania*-infected mice can differentially modulate expression of Fcγ receptors in BMM.

**Introduction**

*Leishmania*, an obligate intracellular parasite, establishes infection within vacuoles of host macrophages. For these parasites to survive, they need to escape host lysis factors, e.g. complement, during invasion and resist further intracellular macrophage killing mechanisms. *Leishmania* was shown to enter macrophages by phagocytosis through different receptors dependent on parasite developmental stage and species. These receptors include complement receptors, Fc receptors and the mannose receptor (reviewed in (20)). Fc receptors are a group of macrophage surface receptors, also found on other cell types including B cells, which have been shown to be used by antibody opsonized *Leishmania* for macrophage invasion (14, 4) and evasion of macrophage activation (13, 15, 2, 17).

Fc receptors are responsible for recognition of the Fc portion of antibodies. Specific Fc receptors recognize a particular antibody class and isotype with different affinities and avidities (11). Aggregation of these receptors after binding antibody triggers phagocytosis of antibody-bound antigen, including antibody opsonized *Leishmania* (8). Receptor aggregation can also induce modulation of macrophage function the characteristic of which are
determined by the type of antibodies associated with antigen and the receptors subtype present on the macrophages. For example CD64, FcγRI, can induce macrophage activation through a cytoplasmic ITAM motif of the receptor after binding to IgG2a with high affinity, while CD32, FcγRIIb, has an inhibitory ITIM motifs which binds to IgG1 antibody isotype and inhibits macrophage activation, but still can promote antibody bound antigen phagocytosis (5, 18). To date, the role of Fc receptors in immunity to Leishmania infection has mostly been studied via genetically modified knockout mice and the direct effect of infection on expression of these receptors has not yet been determined.

The varying macrophage responses which depend on antibody subtype and particularly Fcγ receptor expression may explain the result that although antibody has been shown to play a role in limiting the immune response to L. major other studies indicate a role for antibodies in promoting macrophage activation and killing of Leishmania. Monoclonal antibody raised against Leishmania spp. was shown to induce protection in vitro against L. major promastigotes and in vivo against both L. amazonensis and L. major parasites (1, 7). Activation of Leishmania infected macrophages with IgG to ligate Fc receptors results in superoxide and hydrogen peroxide production and in the presence of IFN-γ, nitric oxide is produced (6).

In this work we extend previous studies in our laboratory showing that LN cells from L. major infected mice can reduce the infection rate of BMM infected with L. amazonensis amastigotes and that this reduction was dependent on antigen specific CD4+ T cells, B cells and IgG. Here we examined whether immune serum could replace B cells and together with antigen responsive CD4+ T cells from L. major infected mice induce macrophage activation
to kill intracellular *L. amazonensis* infection. In this work, immune-serum from healed *L. major* infected mice did not promote a reduction in the infection rate in *L. amazonensis* infected BMM. While macrophage infection with either *L. amazonensis* or *L. major* induced reduction in CD64 mRNA expression compared to non-infected BMM, CD32 mRNA expression was upregulated. However, LN cells from *L. major* infected mice induced a reduction in CD32 expression. These findings suggest a role for *Leishmania* infection and host LN cells in modulation of Fc receptor expression in BMM which could influence productivity of host response.

2. Material and Methods:

*Mice*

C3HeB/FeJ (C3H) and C3Smn.CB17-Prkda<sup>−/−</sup>/J (C3H scid) mice were obtained from Jackson Laboratory (Bar Harbor, Maine). The mice were maintained in a specific-pathogen-free facility. Mice either used for the lymph nodes experiments, serum, or propagation of lesion derived amastigotes (C3H SCID) were infected with 5x10<sup>6</sup> stationary-phase promastigotes in 50 μl of PBS in the left hind footpad. All procedures involving animals were approved by the Committee on Animal Care at Iowa State University.

*Parasites*

*Leishmania major* (MHOM/IL/80/Freidlin) and *Leishmania amazonensis* (MHOM/BR/00/LTB0016) promastigotes were grown to stationary phase in Grace’s insect culture medium (Technologies, Gaithersburg, MD) with 20% heat-inactivated FCS, 2 mM
glutamine, 100 U penicillin per ml, and 100 μg of streptomycin per ml. Amastigotes used for infection were tissue derived and harvested from lesions of C3H SCID mice as described (19). Freeze-thawed *Leishmania* antigen (Ag) was obtained from stationary-phase promastigotes as described in (12).

*Cells and cell culture*

Cells were obtained from bone marrow of the femur and tibia (15-20 x 10⁶ cells) and plated in a 150 x 15 mm Petri dish with 30 ml of macrophage medium containing 30% L-cell conditioned medium, 20% FCS and 50% Dulbecco’s modification of Eagle’s medium (DMEM), 2 mM glutamine, 100 U penicillin per ml, 100 μg of streptomycin per ml and 1 mM sodium pyruvate at 37 °C and 4% CO₂. After 2 days another 20 ml of macrophage medium was added to each dish. At day 6 non-adherent cells were removed by washing the plate two times with PBS and then placing the plates on ice for 20 min and scraping off the remaining adherent cell population. Live cells were counted using trypan blue exclusion and resuspended in complete tissue culture medium (CTCM) containing DMEM, 10% FCS, 2 mM glutamine, 100 U penicillin per ml, 100 μg of streptomycin per ml, 25 mM 4(2-Hydroxyethyl)piperazine-1-ethane sulfonic acid (HEPES), and 0.05 μM 2-β-mercaptoethanol.

Lymph node cells were obtained from LN draining mice infected for 4 weeks with either *L. amazonensis* and *L. major*. Poplitial, inguinal and prescapular lymph nodes were collected from non-infected mice. LN from 4-10 mice were pooled in 2 ml CTCM and grinded with 2 ml tissue grinder and washed with 10 ml CTCM at 300 G, 4 °C for 10 min. LN cells
were re-suspended in 4 ml CTCM and passed through 40 µm nylon cell strainer (BD Falcon, Bedford, MA). Lymph nodes cells were counted using trypan blue exclusion.

2.4 Serum

Blood was collected from C3H mice infected with *L. major* for 12 weeks. Serum was collected by incubating the blood at 4 °C overnight then centrifugating at 450xg for 10 min. Serum stored at 4 °C until used.

*Macrophage infection and cells addition.*

BMM were plated in 24-well plates with glass cover-slips at a rate of 5×10^5 cells/well in 1 ml CTCM. After 24 h BMM were infected with either *L. major* or *L. amazonensis* amastigotes at a 3:1 ratio and incubated at 34°C for 24 h. Wells were washed two times with DMEM to remove extracellular amastigotes and 500 µl of CTCM was added. Lymph node cells were placed with Ag (50 µg/ml) either directly on BMM or in 0.4 µm in diameter transwell (Corning Costar, NY) to separate them from the macrophages (for RT-PCR experiments). Volume was adjusted to 800 µl in the wells and 150 µl in the transwell with CTCM. Antigen was added in the upper chamber with the LN cells. 15 µl serum with 50 µg Ag and 100U IFNγ were added to indicated well with infected macrophages. This mix has incubated together for 5 days at 34 °C, 5% CO₂.

* Determination of the infection rate of macrophages and parasite count
Each individual coverslip was counted via light microscope by examining 3 areas at 100x. In each area, 100 macrophages were examined and the number of infected macrophages and the number of parasites in each macrophage were counted. The average of the 3 areas was used to determine percent of infected macrophages and number of parasites per macrophage on each coverslip.

**RT-PCR.**

Either BMM macrophages infected for 24 hours or exposed to LN cells in the transwell system was used for total RNA extraction using Rapid Total RNA purification system (Marligen Biosciences, Ijamsville, Md.) according to the manufacturer's protocol. RNA concentration was measured spectrophotometrically; RNA samples were then treated with 1.5 U of DNase I (Pierce, Milwaukee, Wis.) per µg of RNA for 45 min at 37°C and 1 µg of each DNase I-treated RNA sample was reverse transcribed in 40-µl reaction volumes with 1X Moloney murine leukemia virus reaction buffer (Promega), 100 U of Moloney murine leukemia virus reverse transcriptase (Promega), 20 U of RNase inhibitor (Applied Biosystems, Foster City, Calif.), 2.5 µM random hexamers (Amersham Biosciences Corp., Piscataway, N.J.) and 0.5 mM each of the four deoxynucleoside triphosphates (USB Corporation, Cleveland, Ohio). RNA samples and random hexamers were incubated together at 70°C for 3 min and room temperature for 10 min. The reverse transcription reaction was performed at 42°C for 1 h; samples were then heated at 95°C for 5 min and incubated on ice for 10 min. Real-time PCR experiments were run on the ABI Prism 5700 sequence detection system (Applied Biosystems, Foster City, Calif.). Each 25-µl reaction contained 0.5 µl of cDNA and 1X ABsolute QPCR Mix (Abgene, Surrey, United Kingdom).
Forward and reverse primers were used at either 300 nM (IDT, Coralville, Iowa) and TaqMan probe for GAPDH labeled with the reporter dye 6-carboxyfluorescein and the quencher dye 6-carboxytetramethylrhodamine (Applied Biosystems, Foster City, Calif.) were used at either 75 or 100 nM. Primer pairs and probes were designed with Primer Express 1.5 software (Applied Biosystems) and are listed in Table 1. For quantitative analysis of gene expression, normalized to an endogenous control, we used the standard curve method, which was detailed previously (10). Briefly, control cDNA samples were serially diluted to obtain standard curves for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA and each target cDNA. For each assay, the resultant cycle threshold (Ct) values were plotted against the input of RNA. For all experimental samples, the cDNA quantity for the target of interest was then determined from its standard curve and normalized to GAPDH control.

Table 1. Sequences of primers and probe used for RT-PCR.

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<th>Target gene</th>
<th>GenBank accession no.</th>
<th>Type</th>
<th>Sequence (5'→3')</th>
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<td>M32599</td>
<td>F</td>
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Statistical analysis.

The statistical significance between different treatments was calculated using student t-test.
Results

Immune serum from L. major infected mice does not induce a reduction in infection rate of L. amazonensis within macrophage.

B cells and CD4+ T cells from L. major infected mice were found, together, (16) to be necessary to induce macrophage activation and reduce the rate of L. amazonensis infection within BMM. This reduction was at least in part antibody dependent. This triad of T cells/B cell and antibody has leaded us to question whether the immunoglobulin and IFNγ alone are sufficient for macrophage activation. To test this hypothesis, immune serum from L. major infected mice for 12 weeks was added to L. amazonensis infected BMM either alone or with IFNγ in the presence of antigen and incubated for 5 days. Serum with IFNγ did not promote a reduction in the infection rate compared to total DLN cells from L. major infected mice (Fig. 1). Even when immune serum was mixed with CD4+ T cells from L. major infected mice, still, no reduction in the infection rate was observed (Fig 1). These results indicate that additional B cells and CD4+ T cells derived immune factors, beyond immunoglobulin and IFNγ, contribute to the effective macrophage response against L. amazonensis.

Fcγ receptors are regulated by Leishmania infection

We have previously demonstrated the necessity of immunoglobulin for macrophage activation during L. amazonensis infection in our in vitro system. The inability of immune serum to induce L. amazonensis killing made us question: 1) Do Leishmania amastigotes influence the expression levels of FcγR and 2) do the total LN cell population of L.
*amazonensis* or *L. major*-infected animals regulate FcγR expression levels of infected macrophages? After 24 h of parasite infection with either *L. amazonensis* or *L. major* amastigotes in BMM, mRNA expression of FcγRI (CD64) and FcγRII (CD32) were measured using real-time RT-PCR and compared to non-infected BMM. Both parasites reduced the expression of CD64 significantly compared to uninfected macrophages (Fig. 2a). In contrast, the infection with either *Leishmania* spp. induced an upregulation of CD32 expression compared to uninfected macrophages (Fig 2b). No significant difference was detected in expression of either Fc receptor (CD64 and CD32) between *L. amazonensis* and *L. major* infection of BMM (Fig. 2a and b). These results indicate that *Leishmania* amastigote infection modulate BMM mRNA expression of Fcγ receptors.

*LN cells from Leishmania infected mice differentially modulated the expression of Fcγ receptors within infected BMM.*

The ability of the total LN cells to induce a reduction in infection rate in an antibody dependent manner, while the immune serum did not, suggested that LN cells from *L. major* infected animals may modulate Fc receptor expression to enable enhanced cellular response to antibody. To explore this, draining LN cells from mice infected for 4 weeks with either *L. amazonensis* or *L. major* were added alone with antigen in a transwell system separated from the macrophages infected with *L. amazonensis* amastigotes. After 5 days incubation, macrophages were analyzed for levels of mRNA expression of CD64 and CD32. No significant difference in the expression of CD64 was found between macrophages exposed to either *L. amazonensis* or *L. major* LN cells (Fig 3a), however macrophages exposed to LN
cells from *L. major* infected mice induced a significant reduction in mRNA expression of CD32 in comparison to *L. amazonensis* LN cells (Fig 3b). These results may indicate that LN cells from *L. major* infected mice secrete factor(s) which reduce expression of CD32.

**Discussion**

In this work we report for the first time the capability of *Leishmania* amastigotes to induce modulation of Fcγ receptor mRNA expression in BMM in vitro. DLN cells from *L. major* infected mice also induced downregulation of CD32 expression in *L. amazonensis* infected BMM. While lesion derived *L. amazonensis* or *L. major* amastigotes coated with antibody induced IL-10 production from macrophages (13), *L. major* promastigotes coated with *Leishmania* monoclonal antibodies produced by specific hybridoma cells were killed by macrophages after infection (9). These monoclonal antibodies protected mice from developing lesions in vivo when injected together with *L. major* or *L. amazonensis* promastigotes (7, 1). Antibody and Fcγ have been shown to be necessary for parasites of *L. mexicana* complex to enter macrophages in vivo (14). Ligation of Fc receptors with IgG induced activation of macrophages to kill *Leishmania* via a PKC dependent pathway (6). These disparate findings demonstrate the differences in response to antibody with various *Leishmania* spp., which may be due to differences in both antibody and Fc receptor type involved in these various experimental systems. In our previous work with LN cells from *L. major* infected mice we demonstrated that activation of BMM and subsequent killing of *L. amazonensis* amastigotes was antibody dependent. In this current study, we could not recover
killing of *L. amazonensis* in BMM by adding either immune serum alone (data not shown) or immune serum with IFNγ (Fig. 1). This suggested that there are other factor(s) produced from B cells and/or CD4⁺ T cells, in addition to antibody and IFN-γ, that help inducing activation of macrophages to kill *L. amazonensis*. To begin to understand this phenomena and given the central role of antibodies in our experimental system, we hypothesize that both infection itself and DLN cells of *L. major* infected animals may influence the expression pattern of Fcγ receptors.

Involvement of Fc receptor in resistance both Old World and New World species of *Leishmania* was demonstrated when susceptible strains of mice became resistant after deletion of Fc receptors (2, 15, 17). Therefore the first time we show that *Leishmania* infection influences expression of these receptors in vitro. *L. amazonensis* was found to induce down-regulation of CD64 expression (Fc activation receptor) and upregulation of CD32 (Fc inhibitory receptor). This modulation of FcR is a technique utilized by other Kinatoplastidea as well; *Trypanosoma cruzi* has been shown also to upregulate expression of Fc receptors II/III (3). Addition of lymph nodes cells from either *L. amazonensis* or *L. major* infected mice did not promote a difference in the expression of CD64 (Fig 3a), which might suggest that both LN cell types can upregulate the expression of this receptor as either IL-10 or IFN-γ can also induce this upreglation (reviewed in (8)). In contrast, *L. major* LN cells significantly downregulate CD32 expression (Fig 3b) as compared to *L. amazonensis* LN cells. We propose that this downregulation may have a role in reducing the inhibitory signal to macrophages and allow macrophage activation through CD64 signaling producing superoxide needed to kill intracellular *L. amazonensis* the parasite. Factor(s) which induce
this downregulation has not been determined, but could be produced from either B cells or CD4+ T cells, or more likely a combination of both.

We propose a model for the mechanism to activate macrophages to control *L. amazonensis* infection needs, both a proper balance in Fc receptors where CD64 expression is upregulated and CD32 expression is downregulated. At the same time specific antibody response, specifically IgG2a binds to CD64, induces activation of macrophages who then produce superoxide in the presence of nitric oxide and kill intracellular *L. amazonensis*.

**References**

Figure 1. Immune serum from *L. major* infected mice does not induce macrophage killing of *L. amazonensis* amastigotes. DLN cells from mice infected with *L. major* for 4 weeks (total LN cells), CD4+ T cells negatively sorted from these cells and/or serum from mice infected with *L. major* for 12 weeks with 100 U IFNγ, were added to BMM infected with *L. amazonensis* amastigotes on cover slips with 50μg/ml antigen and incubated for 5 days at 34 °C. Reduction % infected macrophage is equal to (1- %infected macrophages treatment / %infected macrophages with *L. amazonensis* LN cell)*100. Error bars represent standard error. The data is an average of three separate experiments.
Figure 2. *Leishmania* infection modulates expression of Fcγ receptors. BMM were infected with either *L. amazonensis* or *L. major* or left uninfected. mRNA levels of A. CD64 and B. CD32 were measured using real-time RT-PCR and adjusted to GAPDH levels as described in material and methods. Values are expressed as the ratio to target mRNA levels of uninfected macrophages. These data are averages ± SE of three separate experiments.
Figure 3 DLN cells from *Leishmania* infected mice modulate the expression of Fcγ receptors. DLN cells from C3H mice infected with either *L. amazonensis* or *L. major* for 4 weeks were added to an transwell system with Ag as described in materials and methods separated from BMM infected with *L. amazonensis* amastigotes and incubated for 5 days. The mRNA levels of a. CD64 and b. CD32 were measured using RT-PCR and adjusted to GAPDH levels. Values are expressed as relative to levels after the addition *L. amazonensis* LN cells. These data are averages ± SE of three separate experiments.
Chapter 5

General conclusion

The in vivo differential killing of *L. amazonensis* and *L. major* in C3H mice were reflected in vitro using BMM under identical activation conditions. We have shown that this delayed intracellular *L. amazonensis* killing when compared to *L. major*, occurred under conditions of similar levels of NO and iNOS. Even an exogenous source of NO (SNAP), that is sufficient to induce killing to intracellular *L. major* infection, did not have any affect on *L. amazonensis* intracellular survival. Other factors such as increased replication rate and IL-10, were not the major resistance factors of intracellular *L. amazonensis* to macrophage killing. Only when superoxide was induced in the presence of NO, was *L. amazonensis* killed in vitro to a similar level as *L. major*. This results indicates not only the host immune response to the two species of *Leishmania* is different, but also the requirements for their intracellular killing are different, as NO was sufficient alone for *L. major* intracellular killing, whereas *L. amazonensis* killing requires both NO and superoxide.

Intracellular killing of *L. amazonensis* amastigotes inside macrophages was induced by the addition of DLN cells from *L. major* infected mice. This *L. major* antigen-specific DLN cell activation of BMM was induced by a combination of CD4+ T cells and B cells, which is the first time to show a direct role for B cells in macrophage activation to control *Leishmania* infection. The killing of the intracellular *L. amazonensis* infection in this system was dependent on secreted factors including antibody. Once again similar to the previous
study this killing of intracellular *L. amazonensis* amastigotes by the BMM was dependent on superoxide while *L. major* killing was not.

The antibody dependent killing of *L. amazonensis* was not reproducible using immune serum alone or with either IFN-γ or CD4+ T cells from *L. major* cells infected mice. These results indicate that other factors are needed in addition to antibody to induce intracellular parasite killing. The factors needed may be required to induce effective macrophages antibody receptor (FcR) expression and promote macrophage activation and superoxide secretion. *Leishmania* infection of macrophages influenced FcR expression of these receptors by downregulating the mRNA expression of the activator FcγI receptor (CD64) and upregulating the mRNA expression of the inhibitory receptor FcγRII (CD32). It seems that beside antibody production, DLN cells from *L. major* secrete factor(s) to downregulate CD32 mRNA expression and at the same time induces the mRNA expression of CD64 in a balance to allow macrophage activation by antibodies and induction of superoxide to kill intracellular *L. amazonensis* amastigotes.

These studies emphasis the necessity to have superoxide as a key factor, in the presence of nitric oxide, to kill *L. amazonensis* and it is seems that one of the major resistance strategies for *L. amazonensis* survival inside the macrophages is to prevent superoxide production.

As summary for this study, we drew a model for our understanding of the mechanism by which DLN from *L. major* infected mice induce BMM to kill intracellular *L. amazonensis* amastigotes (Diagram 1). We think that the *L. major* antigen is needed to activate *L. major* antigen specific CD4+ T cells and B cells to produce antibodies and, as yet unknown, specific
factors for upregulation CD64 expression and downregulation of CD32. The *L. major* specific antibody (IgG2a) binds CD64 and in the presence of *L. major* antigen that binds to the receptor bound antibodies causes the CD64 to aggregate and signals through their ITAM motifs to activate signaling cascades including PKC and PLC that result in macrophage superoxide production. Superoxide in the presence of nitric oxide subsequently produce peroxynirrite, which is one of the most known potent endogenous cytotoxic factors, which would ultimately kill intracellular *L. amazonensis*.

Diagram 1. Proposed model for the mechanism of DLN cells from *L. major* infected mice induce BMM to kill intracellular *L. amazonensis* amastigotes.
Figure A1. IL-10 is not responsible for the delayed killing of *L. amazonensis* in comparison to *L. major*. BMM from IL-10 deficient and C57BL/6 mice were infected and activated with LPS and IFN-γ as described in Fig. 1. The percent of the macrophages that were infected for each treatment group was determined at the days indicated and the % of the non-activated control was calculated as described in Fig. 1. The data shown are the mean ± standard error from 2 independent experiments with 3 replicates in each. (*= p<0.05 ANOVA, Scheffe's test for paired comparison)
Figure A2. NADPH-oxidase pathway does not contribute to the kinetics of amastigote killing for either *Leishmania major* or *L. amazonensis* infected macrophages. BMM from C57BL/6J and gp91phox deficient mice were infected and activated with LPS and IFN-γ as described in Fig. 1. The percent of macrophages that were infected for each treatment group was determined at the days indicated, and the % of the non-activated controls was calculated as described in Fig. 1. The data shown are the mean ± standard error from 2 independent experiments with 2 replicates in each experiment.
Figure A3. Both Total IgG and IgG2a are lower in cultures with LN cells from *L. amazonensis* infected mice. LN cells and macrophage infection was done as described in chapter 3. The macrophages were infected with *L. amazonensis* LN cells and LN cells from either *L. major* or *L. amazonensis* infected mice and incubated with antigen for 5 days. The levels of Total IgG (a) and IgG2a (b) were determined by immunobloting the supernatant of the cultures with goat anti-mouse IgG HRP or goat anti-mouse IgG2a HRP, respectively. These results are the average of the densotometry of at least 3 experiments with standard error bar.
Figure A4. Flow staining for CD19 in: a. total LN cells, b. CD19+ cells depleted population, and c. negatively selected B cells. The stain used was PE labeled anti-mouse antibody.
Figure A5. Flow staining for CD8 in; a. total LN cells, b. CD8+ cells depleted population, and c. negatively selected CD8+ cells. The stain used was FITC labeled anti-mouse antibody.
a. Isotype control

Figure A6. Flow staining for CD4 in a. total LN cells, b. CD4+ T cells depleted population, and c. negatively selected CD4+ T cells. The stain used was FITC labeled anti-mouse antibody.
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