DISSERTATION

MACROPHAGE RECEPTOR-MEDIATED RECOGNITION, RESPONSE AND TREATMENT OF *LEISHMANIA MAJOR* INFECTION

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ABSTRACT

MACROPHAGE RECEPTOR-MEDIATED RECOGNITION, RESPONSE AND TREATMENT OF *LEISHMANIA MAJOR* INFECTION

We investigate the role of macrophage receptors in the recognition of *Leishmania major* and response to the parasite, focusing on complement receptors (CR). CR1 and CR3 are the main complement receptors on murine macrophages, recognizing specific parasite surface antigens and parasites opsonized by complement component 3 (C3). Utilizing C3-deficient mice, we show a clear role for complement in enhancing parasite infectivity, and recognition of and response to *L. major* by the murine host. In our *in vitro* experiments blocking CR1 and CR3, there is a clear effect of the route of parasite recognition and entry into the macrophage on parasite phagocytosis and cytokine response to infection by *L. major,* although Th1/Th2 bias is unaffected by blocking complement receptors. Using parasite strains lacking in the two most common surface molecules of *L. major* (LPG and gp63), we show a strong role for gp63 in the interaction with C3 and complement receptors, particularly CR3. Lastly, we demonstrate the utility of targeting anti-leishmanial therapeutics in infected hosts to the macrophage through another macrophage receptor, the scavenger receptor.

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INTRODUCTION

The interaction of the innate and adaptive immune systems is an accepted fact of modern immunology, as is the reliance of the adaptive immune system on the innate immune response. Interactions between a pathogen and the innate immune system may profoundly affect the adaptive immune response, via antigen-presenting cells and their products. An excellent example of this is in *Leishmania major* infection, in which certain mouse strains develop a pronounced type 1 T cell (Th1) response and clear the infection and others develop a type 2 T cell (Th2) response and fail to clear the infection, resulting in progressive lesions and death. The interaction between antigen-presenting cells, their cytokines and T cells may have a dramatic influence on the immune response and subsequent type 1/type 2 T cell (Th1/Th2) bias in *L. major*-infected mice.

Members of the genus *Leishmania* are protozoan trypanosomatid parasites that are transmitted by sand flies to vertebrate hosts when the sand fly injects the metacyclic promastigote form of the parasite, along with its saliva, in the course of a blood meal. Injected *Leishmania* are then engulfed by macrophages and replicate and mature to amastigotes within the macrophages, lysing and then infecting other host macrophages and dendritic cells during this phase. Infected cells are then ingested by the sand fly vector during the blood meal, completing the life cycle by allowing subsequent replication and maturation within the sand fly vector (reviewed in Solbach and Laskay, 2000; Handman, 2000).

Most strains of mice (including C57BL/6 and C3H) develop a self-limiting cutaneous disease when subcutaneously injected with *L. major*. This self-limiting response is characterized by the development of a protective Th1-biased T cell response and the production of IL-12, nitric oxide (NO), and IFN-γ (Titus et al., 1984; Launois et al., 1998). IL-12 upregulates production of IFN-γ and downregulates the Th2 response. IFN-γ and NO induce macrophage activation and subsequent destruction of *L. major* in phagocytic cells, leading to resolution of the disease. Therefore, infection in these strains of mice resembles self-limiting cutaneous leishmaniasis in humans (reviewed in Launois et al., 1998; Liew et al., 1999; Locksley et al., 1999; Solbach and Laskay, 2000). This is an excellent model for the development of a Th1 response and the cellular events leading up to that Th1 bias.

Other strains of mice (notably BALB/c) develop a chronic progressive disease that is not self-limiting and involves a Th2 response to *L. major* infection. Within minutes to hours following subcutaneous infection with *L. major*, BALB/c mice produce IL-4 mRNA in response to a single parasite antigen (Ag), LACK (*Leishmania* homolog of receptors for activated C kinase). The increased IL-4 production promotes the expansion of Th2 cell populations, which activate B cells and stimulate the production of antibody (Ab). However, since *L. major* resides within macrophages, the Ab is not able to bind to and destroy parasites (reviewed in Launois et al., 1998). IL-4 production may also cause downregulation of IL-12 receptors on Th1 cells, resulting in unresponsiveness to IL-12 and inhibition of IFN-γ and NO production by macrophages and NK cells (Vouldoukis et al., 1997). This inhibition results in decreased macrophage activation and decreased clearance of *L. major* from macrophages. Activated Th2 cells also produce IL-10, which

inhibits macrophage activation. With inhibition of macrophage activation and parasite clearance, *L. major* is protected within macrophages and can replicate freely. Thus, *L. major* infection leads to eventual death in these strains of mice, much as can occur in humans infected with *L. donovani*. This model of the Th2 bias provides an excellent contrast for the resistant, Th1 biased, mouse model, suggesting that in identical infections, some form of interaction with the host immune system and some change in the cascade of immune reactions predisposes animals to either clearance (via a Th1 response) or susceptibility (via a Th2 response).

The influence of antigen-presenting cells such as dendritic cells and Langerhans cells on the development of a Th1/Th2 response is well documented. However, the interaction between the *Leishmania* parasite and the macrophages that are the primary phagocytic cells and one of the important antigen-presenting cell populations it encounters in the vertebrate host is not as well characterized. The route of entry and recognition of intracellular pathogens, including bacteria and protozoa, has been shown to influence the subsequent innate immune response to the pathogen (Titus et al., 1994; Brightbill et al., 1999; Re and Strominger, 2001). Therefore, the route of entry of *L. major* into the macrophage and recognition of the parasite by the macrophage may deeply influence the subsequent response of the macrophage to the parasite and the resultant Th1/Th2 response developed by the host. These responses would be dependent on a wide variety of macrophage cell surface receptors, including complement receptors, mannose-fucose receptors, macrophage scavenger receptors, and Toll-like receptors (TLRs), as well as the molecules present on the surface of the *L. major* parasite.

Leishmania major in natural infection is introduced to the mammalian host along with the saliva of the sandfly (*Phlebotomus* sp. or *Lutzomyia* sp.), the vector for *L. major*. While sandfly saliva is not a focus of this work, it is important to consider the effect that the presence of saliva may have on the course of infection and immune response to *L. major*. Sandfly salivary gland lysates have been shown to exacerbate the infectivity of *L. major* and increase lesion size and parasite numbers in infected mice (Titus and Ribeiro, 1988). Salivary gland lysates have also been shown to downregulate Th1-type responses and upregulate Th2-type responses in infected mice (Mbow et al., 1998). Salivary gland lysates did not, however, alter the long-term resistance to *L. major* seen in resistant strains of mice (Theodos et al., 1991). Sandfly saliva has significant immunosuppressive effects on macrophages, reducing nitric oxide production, macrophage activation, and *Leishmania* killing (Hall and Titus, 1995; Theodos and Titus, 1993; Waitumbi and Warburg, 1998), while increasing phagocytosis of *Leishmania in vitro* (Zer et al., 2001). Sandfly saliva has been shown to have potent anti-complement effects, thus potentially modifying another portion of the acute phase response to *Leishmania* (Calvacante et al., 2003). Sandfly saliva has many components that affect host response, including maxadilan, a potent immunomodulatory and vasodilating peptide (Lerner et al., 1991; Grevelink et al., 1995). Maxadilan has been shown to inhibit T-cell proliferation, development of mixed lymphocyte reactions, and development of delayed–type hypersensitivity reactions (Qureshi et al., 1996). Maxadilan also inhibits TNF-α, NO, and IFN-γ production and increases IL-6 and IL-10 production in mouse macrophages (Soares et al., 1998; Titus et al., 2006). Finally, recombinant maxadilan peptide has been shown to exacerbate disease in vivo and vaccination against maxadilan has a protective

effect against *L. major* infection in mice (Morris et al., 2001). Additionally, the vasodilative nature of maxadilan may influence the host response to *L. major* by introducing larger amounts of blood-borne components of innate immunity, such as complement components, fibrinogen, and other acute phase proteins. As many of these acute response components are co-opted by *Leishmania* for enhanced phagocytosis and entrance into macrophages, this vasodilation is potentially another route of exacerbating the infection. In this series of experiments, we chose to investigate the interaction between the parasite and the host without adding in the potent and potentially obscuring effects of the sandfly saliva, but the interaction between the parasite, the host and the co-injected sandfly saliva is an important avenue for further investigation.

Macrophage response to *Leishmania* and other protozoan parasites is a complex interaction, incorporating both very general and nonspecific interactions and extremely specific responses. In general, macrophage responses, and those of other phagocytic cells, can be characterized by phagocytosis and/or cytokine and chemokine production. Phagocytosis consists of several steps, beginning with recognition and attachment of organisms or antigens, engulfment of the foreign material, and degradation of the material or killing of the organism. It is the recognition and attachment phase that is of interest, particularly in terms of downstream signaling and control of adaptive responses. Initially phagocytosis of *Leishmania* occurs at the site of infection, with tissue macrophages recognizing the parasite through a wide range of receptors, including scavenger receptors, mannose receptors, complement receptors, and toll-like receptors (Mosser and Miles, 2007; Tuon et al., 2008; Gomes et al., 2009). These receptors may respond directly to parasite components such as LPG, gp63, parasite DNA, and other cell

surface molecules or may respond to opsonized parasites coated with complement components (C3b, C5a, iC3b, etc.), albumin, fibrin, or other serum-derived acute phase proteins (Vannier-Santos et al., 2002; Mosser and Miles, 2007). However, all of these interactions occur in a milieu of multiple macrophage surface receptors and multiple binding molecules on the parasite surface, any of which may affect the response to the parasite, both initially by the macrophage and downstream by effector cells of the adaptive arm of the immune response. Macrophages have been closely linked to the recognition, response, and survival of a wide array of protozoan parasites, including *Leishmania*, and may serve as an immunologically privileged site for pathogenic organisms that are capable of controlling respiratory burst and other macrophage responses to phagocytosed pathogens (Mosser and Miles, 2007). Of particular interest to our work is the degree to which the initial recognition receptor available to *L. major* controls further macrophage responses and activation of adaptive responses.

When the parasite is injected into the vertebrate host, it is exposed to many components of the immune system, which can destroy the parasite. Included are acute phase proteins, neutrophils, natural killer (NK) cells, macrophages and complement. In order for *L. major* to survive within a vertebrate host, it must be phagocytized by macrophages and thus gain a degree of immunological protection from the other components of the innate immune response. *L. major* phagocytosis is enhanced *in vitro* by complement opsonization, suggesting that complement is utilized by the parasite to increase the infection rate of macrophages (Mosser and Edelson, 1985; Puentes et al., 1988; Mosser et al., 1992; Brittingham and Mosser, 1996; Rosenthal et al., 1996). The initial complement response to *L. major* occurs primarily through the alternative

complement pathway, in the absence of antibody, with opsonization of parasites by C3b following cleavage of C3 by C3 convertase or by iC3b following cleavage of C3 by gp63. Opsonization of the parasite produces enhanced phagocytosis, but may also influence the recognition and attachment site of the parasite to the macrophage, thus affecting the subsequent course of the overall immune response to the parasite. The complement cascade does not only end in opsonization of pathogens, but also leads to formation of membrane attack complexes (MACs) on opsonized parasites. These MACs promote pathogen lysis and destruction by creating holes in the external membrane and disrupting the osmotic balance of the parasite. *L. major* appears to have at least two membrane structures, LPG and gp63, that may mitigate the effects of complement binding and divert the formation of MACs (Mosser and Brittingham, 1997). The effects of these two membrane structures on complement and macrophage recognition and response to *L. major* may significantly influence the course of the overall immune response to the parasite.

Dendritic cells are important phagocytic cells in the interaction between the innate and adaptive immune response. *In vivo*, dendritic cells are recruited following the first, "silent" period of infection and are key players in the activation and immigration of T cells, production of IL-12, parasite killing, and eventual resolution of the lesion (Berberich et al. 2003; von Stebut, 2007). Dendritic cells are potent antigen presenting cells and producers of IL-12, Il-27, IL-23, IL-1 and other related pro-inflammatory cytokines, with subsequent NK cell activation and generally formation of a Th1 response (Tuon et al., 2008). Repeatedly, dendritic cells have been shown to have a powerful effect on the induction of adaptive T-cell responses. Although macrophages may

produce some level of IL-12, they are not the primary source of IL-12 in the lymphoid tissues, with dendritic cells serving in that role (von Stebut, 2007). However, dendritic cells are primarily involved in the response to amastigotes, rather than promastigotes, although there is some *in vitro* evidence that promastigotes can also activate dendritic cells (Abou Fakher et al., 2009). Although dendritic cells have been shown to be the primary cells involved in the long-term response to *L. major,* after the initial infection has occurred (von Stebut, 2007), macrophages are the focus of this dissertation. The initial phagocytosis and response to *Leishmania* occurs within the interface between the macrophage and the parasite, and is thus worthy of some attention regarding the role of the parasite surface structures and the macrophage receptors in the overall innate and adaptive immune response to the parasite.

Chapters 1 and 2 - *L. major* Surface Structures

LPG is a long chain glycoconjugate found in abundance on the external membrane of *Leishmania* promastigotes. It is the major cell surface structure on promastigotes, particularly on the infectious metacyclic form, and covers the entire surface of the parasite, including the flagellum (Turco and Descoteaux, 1992). LPG strands are linked to the parasite by a conserved glycosylphosphatidylinositol (GPI) anchor and contain a glycan core with a highly variable repeating saccharide-phosphate region and oligosaccharide cap (Turco, 1990). LPG strands, particularly the apical oligosaccharide cap, are attachment sites for complement, enhancing the interaction of C3b with Factor B (Green et al., 1994; Brittingham and Mosser 1996). This, coupled with the great abundance of LPG on the surface of the parasite, makes it an excellent C3 opsonization site.

During metacyclogenesis, the polysaccharide region becomes greatly elongated (up to double in *L. major*), lengthening the LPG molecule (Sacks et al., 1990). The increased length of LPG on metacyclic promastigotes provides a degree of protection from complement-mediated lysis, as metacyclic promastigotes are more resistant to lysis than procyclic ones (Puentes et al., 1988). The extension of LPG prevents effective formation of the MACs, keeping them away from the vulnerable parasite membrane. When MACs form at a distance from the parasite membrane, this results in shedding of the C5b-C9 MAC complexes while still allowing opsonization of the parasite by C3b (Puentes et al., 1988; Puentes et al., 1990).

LPG is not generally thought to have a significant role in non-opsonized binding to macrophages *in vivo* (Mosser and Brittingham, 1997), despite the evidence for *in vitro* non-opsonized binding (Talamas-Rohana et al., 1990). The abundance and importance of LPG as a surface molecule for opsonization by C3 and subsequent CR1 binding to macrophages argues that it may indeed be an important link in the entry of the parasite into macrophages, initiating the innate immune response and subsequent formation of the Th1/Th2 bias.

The most abundant protein on the surface of *Leishmania* promastigotes is a 63 kDa zinc metalloprotease called gp63, or leishmanolysin (Etges et al., 1986), which is attached to the parasite membrane with a glycosylphosphatidylinositol (GPI) linkage (Bordier et al., 1986). Gp63 has a strong ability to degrade a wide variety of proteins, many of which are involved in opsonization and the innate immune response, including albumin, casein, immunoglobulin, hemoglobin, and complement proteins (Chaudhuri et al., 1988). Additionally, like LPG, gp63 serves as a C3 acceptor, supporting opsonization

by C3 and thus mediating the attachment of promastigotes to macrophages. It also regulates complement-mediated lysis by proteolytic degradation and inactivation of C3b into a form similar to that of inactivated C3b (iC3b) (Brittingham and Mosser, 1996). This iC3b-like form remains opsonic, binding to gp63 and LPG on the parasite surface, but does not allow for continuation of the complement cascade and deposition of MACs. As well as its role as a C3 acceptor, gp63 may also serve as an independent ligand for attachment of *Leishmania* to macrophages, via the fibronectin receptor and CR3 (Mosser and Brittingham, 1997, Brittingham et al., 1999). Binding of of promastigotes to macrophages is enhanced in parasites with gp63, although the mechanism is not fully elucidated (Mosser and Brittingham, 1997). Gp63-driven enhancement of phagocyte binding may affect the interactions between *L. major* and macrophages and thus the resulting Th1/Th2 bias.

Two strains of genetically modified parasites allow investigation of these surface molecules: *L. major* parasites that are deficient in LPG expression (LPG^-) (Späth et al., 2000; Späth et al., 2003) and *L. major* parasites that have genes 1-7 of the gp63 gene family deleted ($gp63^{-(1-7)}$) (Joshi et al., 2002). The LPG^- parasites were created by deleting the *L. major* Golgi guanosine diphosphate-mannose transporter, LPG2, by homologous replacement of the entire LPG2-coding region in LV39 wild-type parasites (Späth et al., 2003). This produced parasites unable to manufacture the full LPG or proteophosphoglycans (PPG), even though able to create their precursors. Small surface GIPLs (glycosylinositol phospholipids) and the glycosylphosphatidylinositol (GPI) anchored gp63 and gp46 molecules remained intact and fully expressed (Späth et al., 2003). $Gp63^{-(1-7)}$ parasites, on the other hand, were created by targeted replacement of

the specific 24 Kb region encoding for gp63 genes 1-7 with drug resistance genes *hyg* or *sat*, resulting in deletion of all 7 of the gp63 genes and parasites unable to express any component of gp63 (Joshi et al., 2002). Both LPG^- and $gp63^{-(1-7)}$ parasites have greatly reduced virulence but still infect normal susceptible mice (Späth et al. 2000, Späth et al. 2003, Joshi et al. 2002). Infections with these modified parasites are initially attenuated and exhibit a delayed onset of lesions. For both of these parasites, entry into susceptible BALB/c murine macrophages does not appear to be disrupted (Späth et al., 2000; Joshi et al., 2002), suggesting that macrophage receptors governing entry of the parasite into the macrophage are not universally effected by the removal of these surface molecules. However, neither of these groups investigated the role of LPG or gp63 on the development or maintenance of the Th1/Th2 bias or in conjunction with complement and complement receptors.

Chapter 2 - Macrophage Receptors

In natural infection, sand flies inject the infectious metacyclic form of *L. major* into the skin of the mammalian host, where phagocytic cells engulf it. Macrophages are the principal phagocytic cell for *L. major* and are involved in stimulation of the T cell response to the parasite. Phagocytosis of pathogens is key in order to provide antigens for presentation to effector cells. Prior to phagocytosis, parasites would encounter complement, acute phase proteins, and other components of the innate immune system in the tissue spaces. These components of innate immunity are involved in enhancing the recognition and phagocytosis of *L. major* by macrophages (Mosser and Edelson, 1984; Mosser et al., 1992). Phagocytic macrophages need to recognize pathogens, utilizing a

wide range of surface receptors in doing so. Among these receptors are the complement receptors, TLRs, fibronectin and mannose-fucose receptors.

Complement receptors (CR) involved in *Leishmania* infection include CR1 and CR3. CR1 recognizes and binds to C3b, as well as possibly *Leishmania* LPG (da Silva et al., 1989; Talamas-Rohana et al. 1990; Rosenthal et al., 1996). CR3 binds to the inactive form of C3b (iC3b), LPG, and possibly gp63 (Mosser and Edelson, 1985; Russell and Wright, 1988; Wilson and Pearson, 1988; Rosenthal et al., 1996). CR3 appears to be primarily involved in phagocytosis of procyclic, low infectivity *Leishmania* (Puentes et al., 1988) and amastigote forms (Guy and Belosevic, 1993; Kelleher et al., 1995). There is a reduction in the uptake of *Leishmania* promastigotes when this receptor is blocked (Mosser et al., 1996; Rosenthal et al., 1996).

Da Silva et al. (1989) showed that CR1 (CD35, the receptor for C3b) is involved in the phagocytosis of infectious metacyclic *L. major* by human macrophages, while CR3 (CD11b, the receptor for iC3b) is involved in internalization of the less infectious procyclic form of *L. major*. This result was obtained by blocking either CR1 or CR3 with antibody or by modulating the expression of CR1 or CR3 off the surface of the macrophage, thereby forcing entry of *L. major* through CR1, CR3, or non-complement receptors. Blocking the CR1 receptor did not prevent metacyclics from entering macrophages, but it did always significantly reduce uptake of *L. major*. Entry of *L. major* via the CR1 receptor also induced less of an oxidative burst (da Silva et al., 1989). Blocking the CR3 receptor resulted in slight decreases in *L. major* uptake. Similar work in the mouse and human also implicated the CR3 receptor in parasite uptake (Cooper et al., 1988; Wilson and Pearson, 1988). Thus, it is apparent that *L. major* is recognized by

multiple receptors on antigen-presenting cells (and macrophages in particular) early in infection. However, the effect that blocking/forcing entry of the parasite through one or the other complement receptor or mannose-fucose receptor had on subsequent Th1/Th2 bias was not investigated, nor was the effect of parasite membrane molecules on the uptake via any of the receptors and subsequent Th1/Th2 bias.

Other than complement receptors, there are numerous other receptors on phagocytic cells that are involved in the recognition and phagocytosis of *L. major*. These include Toll-like receptors (TLRs), mannose receptors, scavenger receptors, fucose receptors, and other non-specific receptors on dendritic cells, macrophages, and Langerhan's cells. While the effects of these receptors on anti-leishmanial activity and subsequent Th1/Th2 bias was not specifically addressed in these experiments, the potential for these receptors to be involved in interactions between macrophages and the *L. major* parasite is worth further consideration.

Toll receptors are involved in the response to pathogen–associated molecular patterns (PAMPs), which trigger innate immunity, resulting in the production of antibacterial and antifungal peptides. Currently, as many as 12 TLRs are known in mammalian species (Imler and Hoffmann, 2001; Medzhitov, 2001; Takeuchi and Akira, 2001). The cytoplasmic domain of these TLRs has homology with the IL-1 receptor and they are classified into the IL-1R/TLR superfamily (Imler and Hoffmann, 2001; Medzhitov, 2001). TLRs are activated by molecular patterns that recur amongst pathogens. TLR2 appears to be involved in recognition of many pathogen associated molecules, such as peptidoglycan, bacterial lipoproteins, lipoarabinomannan of *Mycobacterium spp.*, zymosan, glycosylphosphatidylinositol anchors, and glycoinositolphospholipids

(including LPG and gp63), while TLR4 recognizes lipopolysaccharide and lipoteichoic acid from gram-positive bacteria (Medzhitov, 2001). TLRs (specifically TLR2 and TLR4) activate IL-1R-associated kinase (IRAK) and thence the NFκB pathway via MyD88 (reviewed in Imler and Hoffmann, 2001; Takeuchi and Akira, 2001; Takeda et al., 2003). Activation of TLRs may induce several pro-inflammatory cytokines, including IL-1, IL-6, IL-12 and TNF-α, as well as affecting the maturation of dendritic cells and expression of T cell costimulatory molecules (Underhill et al., 1999; Adachi et al., 2001; Kaisho and Akira, 2001; Scanga et al., 2002).

The TLRs and associated molecules such as MyD88 have been shown to be important in recognition and response to *L. major*. Murraille et al. (2003) showed that MyD88 was necessary for the effective development of a protective Th1 response and mice lacking MyD88, despite having a resistant background phenotype, developed non-resolving, progressive lesions from *L. major* infection, with accompanying increased parasite load and cytokine responses similar to susceptible BALB/c mice. This evidence that an essential TLR-pathway effector molecule is necessary for resistance to and clearance of *L. major* infection is paralleled in experiments by other investigators (Hawn et al., 2002; Becker et al., 2003; de Veer et al., 2003; Debus et al., 2003). TLR2 has since been definitively linked with the recognition and response of cells to *Leishmania*, particularly LPG surface molecules. de Veer et al. (2003) demonstrated that LPG not only stimulated MyD88-dependent pathways of cytokine response, but also that it was recognized through TLR2 in human TLR-transfected 293T cells. TLR9 and TLR4 have also been implicated in the protective response to *Leishmania* (Kropf et al., 2004; Liese et al., 2007,

Abou Fakher et al., 2009). The relationship between TLR2 and LPG suggests that other *Leishmania major* surface moieties, such as gp63, may also be recognized by TLRs.

The role of the mannose-fucose and fibronectin receptors in Leishmania major interaction with macrophages is less clear. Although da Silva et al. (1989) did not recognize the mannose-fucose receptor as having a significant role, other research has suggested that it may be involved in *Leishmania* recognition and uptake (Wilson and Pearson, 1988; Green et al., 1994; Chakraborty et al., 2001). The fibronectin receptor has also been implicated in the recognition and enhancement of phagocytosis of *Leishmania* by macrophages, although it does not appear to play a major role (Brittingham et al., 1999). Mannose-fucose receptors may bind to mannose domains found in LPG chains on the surface of *L. major*, serving as an enhancer of CR1 and/or CR3 based phagocytosis (Wilson and Pearson, 1988; Chakraborty et al., 2001). As well, the mannose-fucose receptor may provide a site for parasite recognition and binding in the absence of complement opsonization (Wilson and Pearson, 1988; Chakraborty et al., 2001). The fibronectin receptor, a member of the B1 integrin family, may bind to gp63, promoting adhesion of promastigotes to macrophages and efficient parasite phagocytosis (Soteriadou et al., 1992; Brittingham et al., 1999). In both cases, these receptors are secondary, either enhancing or replacing more efficient CR1 or CR3 complement-dependent receptors (Wilson and Pearson, 1988; Guy and Belosevic, 1993; Brittingham et al., 1999).

The macrophage scavenger receptor is a non-specific group of receptors comprising two related but distinct classes, the Class A scavenger receptors and the Class B scavenger receptors, that serve to recognize foreign or non-self molecules, much as Toll-

like receptors do, although with less specificity. Of particular interest are the class A scavenger receptors, which are involved in phagocytosis of bacteria and other pathogens, phagocytosis of apoptotic and senescent cells, and endocytosis of low density lipoproteins, among other roles (Yamada et al., 1998). Among the molecules recognized by the class A scavenger receptors are albumin, LDLs, HDLs, proteoglycans, lipoteichoic acid, lipopolysaccharide, gram negative and gram positive bacteria, serum amyloid and apoptotic cells. Once the appropriate ligand binds to the scavenger receptor, either endocytosis or phagocytosis ensues, with resultant uptake by the macrophage of the bound particle, molecules or pathogen and eventual fusion with lysosomes for breakdown of the phagocytosed material (Yamada et al., 1998; Platt and Gordon, 2001). This has been shown in experiments targeting the class scavenger receptors, where co-localization of labeled ligands and phagolysosomal compartments is seen (Tempone et al., 2004). Additionally, scavenger receptors are expressed primarily on mature, activated macrophages and appear to be upregulated in expression on macrophages infected with *L. chagasi* (Tempone et al., 2004, Broz et al., 2005). With the ability to recognize proteoglycans and lipopolysaccharides, it is not inconceivable that the scavenger receptors may also recognize elements of the *Leishmania* glycocalyx and thus be involved in phagocytosis of *L. major,* although this is not specifically investigated in this thesis.

Chapter 3 - Targeted Treatment

Our collaborator, Robert Ryan (Children's Hospital Oakland Research Institute), discovered that ampB (as well as, presumably, other hydrophobic drug molecules) can be formulated into discrete, nonliposomal, lipid particles that are stabilized by integrally

associated apolipoproteins. Apolipoproteins function in transport of hydrophobic biomolecules and are recognized by numerous of the macrophage scavenger receptors. A common property shared by these proteins is an ability to disrupt phospholipid bilayer vesicles and transform them into disc shaped lipid/protein complexes (apolipoprotein stabilized disc complexes or ASDC). ASDCs are distinguished from conventional liposomes or lipid microvesicles in that they do not have an aqueous core, they are fully soluble in aqueous media, the diameter of ASDCs is 8-20 nm (rather than 60-250 nm for liposomes), and apolipoproteins are an intrinsic structural element of the ASDC (Narayanaswami et al., 2004). In short, they are more amenable to solublization, more readily lyophilized, smaller and more easily phagocytosed by macrophages, and have an intrinsically attached and readily available ligand for macrophage scavenger receptors.

Targeting to macrophages via various receptors is a method of drug delivery that shows significant promise in the treatment of intra-histiocytic pathogens, including fungi, bacteria, viruses, and protozoa. Among the receptors targeted by various therapeutics is the mannose-fucose receptor on macrophages, utilizing varying mannose domains as ligands (Datta et al., 2003; Medda et al., 2003; Basu and Lala, 2004; Nan et al., 2004), and the Class B and Class A macrophage scavenger receptors; using phosphatidylserine-coated liposomes, a modified maleated bovine serum albumin molecule, polyguanylic acid, and other ligands (Demidova and Hamlin, 2004; Tempone et al., 2004; Broz et al., 2005). All of these methods of targeting, although addressing different receptors and using different ligands, have shown significant promise in terms of their ability to increase uptake of drugs into macrophages. The success of those targeting the macrophage scavenger receptor is of particular interest.

Lipid formulations for delivery of drugs to various phagocytic cells have been quite successful. A wide range of lipid formulations have been used in drug delivery systems, including acetylated LDLs, HDLs, lipid microparticles, lipid complexes, and lipid microvacuoles (Nicolas et al., 1990; Erni et al., 2002; Medda et al., 2003; Basu and Lala, 2004,). Researchers and clinicians have seen decreased drug clearance, decreased toxicity, and increased efficacy of various drugs delivered in association with lipid formulations (Gangneux et al., 1996, Basu and Lala, 2004).

Treatment for leishmaniasis, particularly visceral leishmaniasis, has been principally achieved via the pentavalent antimonial compounds, with full remission and supposed clearance of Leishmania infection in humans, although recurrence is seen in immunosuppressed patients (Singh and Sivakumar, 2004). However, currently, in some endemic areas, there is as much as 64% resistance to these drugs, suggesting that new compounds are important in the front-line defense against *Leishmania* infection (Sundar, 2001). The pentavalent antimonials also have some significant toxic side effects, including cardiotoxicity, pancreatitis, arthralgia and myalgia, pancytopenia and rare renal toxicity (Lee & Hasbun, 2003).

Among the compounds used to replace the pentavalent antimonials is the antifungal and antiprotozoal drug Amphotericin B (AmpB). This is a highly active, insoluble, hydrophobic molecule with some significant toxicity complications, including acute fever, bone pain, chills & cardiac arrest and chronic hypokalemia & nephrotoxicity (Singh and Sivakumar, 2004; Croft and Yardley, 2002). Amp B has an affinity for sterols such as ergosterol and episterol, found in fungal and protozoal organism cell membranes, respectively. Once bound to membrane sterols, the AmpB aggregates and forms pores in

the cell membranes, leading to leakage of cell contents, osmotic imbalance, and cellular death. In the mammalian host, Amp B binds only weakly to cell membranes, as there is reduced affinity to cholesterol, the mammalian cell membrane sterol, although still sufficient affinity to produce toxic effects. With incorporation of the AmpB into liposomes (Ambisome), the toxicity is significantly reduced, coupled with a significant increase in circulation time (Gangneux et al., 1996).

The ASDC-bound AmpB not only has similar characteristics to currently known and available AmpB-lipid compounds such as Ambisome (lipisome-bound AmpB), but also has further potential as a macrophage-specific treatment. The apolipoprotein-A-I stabilizing the ASDCs is a ligand for macrophage Class A scavenger receptors and, as such, will activate the macrophage phagocytosis of the ASDC AmpB complexes. This should, theoretically, lead to enhanced incorporation of ASDCs into mature, activated macrophages, where there is a greater likelihood of them affecting the *L. major* amastigotes causing the activation of the macrophages. This has been seen with phosphatidylserine-grafted liposomes, which bind to some of the Class B and Class A scavenger receptors of macrophages and co-localize with *L. chagasi* amastigotes (Tempone et al., 2004).

Combining experimental *in vitro* blocking of the two primary complement receptors (CR1 and CR3) on macrophages with investigations into the role of complement opsonization *in vivo*, utilizing C3-deficient mice, we show a clear link between the role of complement in opsonization of *L. major*, complement receptors, and parasite infectivity and response to infection by the mouse host. Complement and, by extension, complement receptors, are not required for parasite infectivity and replication, but do

appear to play a large role in early host-parasite interactions. In Chapter 1, using parasite strains lacking in one the two most common surface molecules of *L. major* (LPG and gp63), we show a correlation between gp63 and C3 in *in vivo* experiments, demonstrating similar responses between models where C3 was lacking in the host and models where gp63 was lacking in the parasite. The *in vitro* experiments shown in Chapter 2 demonstrate a similar correlation between models where gp63 was deleted and models where CR3 was blocked, showing that CR3 is likely the primary receptor involved in the recognition of opsonized gp63 on parasites, although CR1-blocking also affected uptake of parasites via gp63. LPG deletion in parasites had an overwhelming effect on *in vivo* and *in vitro* models, with little effect seen from C3 deletion or blocking complement receptors. Th1/Th2 bias was only minimally affected by manipulations of complement receptors or complement opsonization, although there is a transient lag in Th1 response, and concomitant transient Th2 bias, evident in the experiments utilizing C3-deficient hosts or gp63-deficient parasites. Finally, in Chapter 3, utilizing a therapy targeted to macrophages through the macrophage scavenger receptor, we demonstrate that parasite infections in susceptible mice can be targeted and cleared without influencing the Th1/Th2 bias.

CHAPTER 1

THE ROLE OF COMPLEMENT COMPONENT 3 AND THE *LEISHMANIA MAJOR* SURFACE MOIETIES LIPOPHOSPHOGLYCAN AND GP63 ON *IN VIVO* INFECTION OF MICE WITH *L. MAJOR*

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CHAPTER SYNOPSIS

Interaction between the vertebrate host and the protozoan parasite *Leishmania major* may influence the progression of disease and long-term response to the parasite by the host. Interactions between complement, particularly complement component 3 (C3), and the parasite surface molecules LPG and gp63 are of particular interest, as opsonization of parasites may affect recognition, receptor activation, and the subsequent immune response by phagocytic cells and the downstream development of immunity. We utilized C3 knockout (C3KO) mice on a *L. major*-resistant background and LPG2-deficient (LPGKO) and gp63$^{1\text{-}7}$-deficient (gp63KO) parasites to investigate this interaction. Infection of C3KO mice with wild-type *L. major* resulted in increased duration of infection, increased severity of infection, and increased levels of cytokines at certain timepoints. Similar results were seen in resistant mice infected with gp63KO parasites. A slight additive effect was seen with gp63KO parasites infecting C3KO mice, with slightly increased duration and severity of infection. Parasites lacking LPG had no appreciable differences in response between resistant, susceptible, or C3KO mice, with overall infection rates, pathology, and cytokine responses being greatly diminished and indistinguishable between strains. Taken as a whole, these findings suggest that C3, contrary to previous speculation, is not a requirement for resistance to *L. major* infection nor is it a requirement for infection by *L. major*, but it does play an important role in development of the host response to *L. major* infection. Additionally, the *Leishmania* surface molecule gp63 is intimately involved in the interaction between *L. major* and host C3.

INTRODUCTION

Leishmania spp. are significant pathogens of humans and animals throughout much of the tropical and sub-tropical world, with over 300 million people at risk world-wide (WHO, 2000). Additionally, *Leishmania* are pathogens that are intimately associated with the immune system of the host, residing in macrophages and other phagocytic cells and thus they affect the immunity of the host through their interactions with components of the innate immune system, including complement (Puentes et al., 1988; Puentes et al., 1990; Rosenthal et al., 1996; Mosser and Brittingham, 1997; Chakraborty et al., 2001). The interactions between complement and surface molecules of *Leishmania* are potentially of great importance in host recognition of the parasite and the resultant response of the immune system.

Prior to phagocytosis, parasites encounter complement, acute phase proteins, and other components of the innate immune system in the tissue spaces. These components of innate immunity are involved in enhancing the recognition and phagocytosis of *Leishmania major* by macrophages and other phagocytic cells (Mosser and Edelson, 1984; Mosser et al., 1992). Phagocytic cells need to recognize pathogens, utilizing a wide range of surface receptors. Among these receptors are the complement receptors, TLRs, fibronectin and mannose-fucose receptors. Complement receptors (CR) involved in *Leishmania* infection include CR1 and CR3. CR1 recognizes and binds to C3b, as well as possibly *Leishmania* LPG (da Silva et al., 1989; Rosenthal et al., 1996). CR3 binds to the inactive form of C3b (iC3b), LPG, and possibly gp63 (Mosser and Edelson, 1985; Russell and Wright, 1988; Wilson and Pearson, 1988; Talamas-Rohana et al., 1990; Rosenthal et al., 1996) and, in *L. infantum*, parasite surface antigen 2 (Kedzierski et

al., 2004). CR3 appears to be primarily involved in phagocytosis of procyclic, low infectivity *Leishmania* (Puentes et al., 1988) and amastigote forms (Guy and Belosevic, 1993; Kelleher et al., 1995). There is a reduction in the uptake of *Leishmania* promastigotes when this receptor is blocked (Rosenthal et al., 1996; Mosser and Brittingham, 1997), suggesting that this receptor is important for *Leishmania* recognition and uptake by phagocytic cells.

Complement is an important component of the non-specific innate immune response. One of the key elements of the complement cascade is complement component 3 (C3). This component is central to the complement cascade and mediates opsonization of pathogens, as well as downstream elements of the cascade that form the membrane attack complex. C3 interaction with *Leishmania* has been reported to cause complete destruction of *Leishmania* via the complement cascade and membrane attack complex-driven lysis (Dominguez et al., 2003; Moreno et al., 2007). C3 has also been shown to play a role in the progression of cutaneous lesions in *L. major* infection, with lowered levels of C3 secondary to increased C3 consumption being linked to resistance to *L. major* infection and reduced lesion progression (Jacobs et al., 2005). However, C3 has not been fully tested *in vivo* to determine the role that it plays in the interaction between *L. major* and the vertebrate host.

L. major phagocytosis is enhanced *in vitro* by complement opsonization, suggesting that complement is utilized by the parasite to increase the infection rate of macrophages (Mosser and Edelson, 1985; Puentes et al., 1988; Mosser et al., 1992; Brittingham and Mosser, 1996; Rosenthal et al., 1996). The complement cascade does not only end in opsonization of pathogens, but also leads to formation of membrane

attack complexes (MACs) on opsonized parasites. These MACs promote pathogen lysis and destruction by creating holes in the external membrane and disrupting the osmotic balance of the parasite. *L. major* appears to have at least two membrane structures, LPG and gp63, that may mitigate the effects of complement binding and divert the formation of MACs (Mosser and Brittingham, 1997).

Lipophosphoglycan (LPG) is a long chain glycoconjugate found in abundance on the external membrane of *Leishmania* promastigotes. It is the major cell surface structure on promastigotes, particularly on the infectious metacyclic form, and covers the entire surface of the parasite, including the flagellum (Turco and Descoteaux, 1992). LPG strands are linked to the parasite by a conserved glycosylphosphatidylinositol (GPI) anchor and contain a glycan core with a highly variable repeating saccharide-phosphate region and oligosaccharide cap (Turco, 1990). During metacyclogenesis, the polysaccharide region becomes greatly elongated (up to double in *L. major*), lengthening the LPG molecule (Sacks et al., 1990). LPG strands, particularly the apical oligosaccharide cap, are attachment sites for complement, enhancing the interaction of C3b with Factor B (Green et al., 1994; Brittingham and Mosser, 1996). This, coupled with the great abundance of LPG on the surface of the parasite, makes it an excellent C3 opsonization site. Additionally, LPG may serve for an independent ligand for phagocytic cells, recognized by complement receptors and Toll-like receptors (Blackwell et al., 1985; Talamas-Rohana et al., 1990; Descoteaux and Turco, 1999).

The most abundant protein on the surface of *Leishmania* promastigotes is a 63 kDa glycoprotein called gp63, or leishmanolysin (Etges et al., 1986). Gp63 expression is increased markedly as promastigotes progress into metacyclogenesis and become more

infectious (Mosser and Brittingham, 1997). Gp63 is a zinc metalloprotease with a strong ability to degrade a wide variety of proteins, including albumin, casein, immunoglobulin, hemoglobin, and complement proteins (Chaudhuri and Chang, 1988). It is attached to the parasite membrane with a glycosylphosphatidylinositol (GPI) linkage (Bordier et al., 1986). Like LPG, gp63 serves as a C3 acceptor, supporting opsonization by C3 and mediating the attachment of promastigotes to macrophages. It also regulates complement-mediated lysis by proteolytic inactivation of C3b into a form similar to that of inactivated C3b (iC3b) (Brittingham and Mosser, 1996). This iC3b-like form remains opsonic, but does not allow for continuation of the complement cascade and deposition of MACs. As well as its role as a C3 acceptor, gp63 may also serve as a ligand for attachment of *Leishmania* to macrophages, via the fibronectin receptors and Mac-1 receptor (CR3) (Mosser and Brittingham, 1997; Brittingham et al., 1999). What is clear is that the presence of gp63 does enhance the binding of promastigotes to macrophages, even if the mechanism is not fully elucidated (Mosser and Brittingham, 1997). This enhancement of binding and use of alternate receptors may affect the interactions between *L. major* and macrophages and thus the resulting nature of the specific immune response to the parasite.

Here we report on experiments aimed at exploring the role of complement on uptake and maintenance of a *L. major* infection. These were done in order to evaluate the need, or lack thereof, for complement-associated phagocytosis of *L. major* in the establishment and maintenance of an infection, as well as to investigate differences in cytokine expression in the absence of complement opsonization. Mice with the C3 gene deleted on a resistant background were utilized. This removes C3, the main complement

component associated with opsonization and recognition by complement receptors on macrophages. With this reduction in C3, we hoped to elucidate the effect of C3 and its interactions with parasite surface protein gp63 and parasite LPG on the phagocytosis and eventual survival of *L. major* within the host, as well as the nature of the immune response to the parasite.

METHODS

Mice

Young adult female BALB/c, C57BL/6, and C3KO mice were bred at the Laboratory Animal facility at Colorado State University or were purchased from the National Cancer Institute (Frederick, MD) or Jackson Laboratories (Bar Harbor, ME). Mice were kept in colonies at Colorado State University, under supervision of the CSU Laboratory Animal Resources Department with authorization by the Animal Care and Use Committee. Experiments complied with all relevant federal guidelines and institutional policies.

Mice were anesthetized with ketamine and xylazine prior to subcutaneous inoculation with 1 x 10^6 *L. major* parasites in 50μL of Dulbecco's modified Eagle Medium wash [DMEM (Sigma-Aldrich, St. Louis, MO), 10mM Hepes (Sigma-Aldrich), 100U/mL penicillin (Gibco, Carlsbad, CA), 100μg/mL streptomycin (Gibco), 2mM L-glutamine (Gibco), 1mM sodium pyruvate (Gibco), 0.2mM L-asparagine (Calbiochem, San Diego, CA), 0.6mM L-arginine (Calbiochem)] in the left hind footpad. Footpad lesion size was measured using a Vernier caliper at 2 - 4 day intervals throughout a given experiment. For measurement of lesion size the infected left footpad was compared to the normal right footpad. Mice were euthanized in a CO_2 chamber at designated timepoints to assess parasite burden and cytokine responses of re-stimulated lymph node cells. Animals in all groups were euthanized if they reached a predetermined level of lesion severity and/or displayed clinical signs of distress or pain.

Parasites

L. major parasites from the LV39 MRHO/Sv/59/P (wild type, WT) and gp63$^{1-7}$ knockout (gp63KO) (Joshi et al., 1998; Joshi et al., 2002) strains were propagated on sheep blood agar in parasite growth medium [RPMI-1640 cell culture medium (Sigma-Aldrich, St. Louis, MO) with 5% heat-inactivated fetal bovine serum (FBS) (Hyclone, Logan, UT), 10mM Hepes, 100U/mL penicillin, 100μg/mL streptomycin, 2mM L-glutamine, 1mM sodium pyruvate, 0.2mM L-asparagine, 0.6mM L-arginine, and 2% sterile-filtered normal human urine]. *L. major* parasites from the LPG2^{-} strain (LPGKO) (Späth et al., 2003) were grown on sheep blood agar in identical medium, with the addition of 15 μg/mL of hygromycin (Gibco). Parasites were passaged through mice (susceptible BALB/c mice were infected with parasites, lesions were allowed to develop, then parasites were isolated from the lesions) every 2 months to retain virulence and from flask to flask twice a week. At 5 - 6 days after flask inoculation, parasites were removed from the growth medium, centrifuged to remove dead parasites and debris, washed in DMEM wash [as described above] and counted on a Neubauer hematocytometer prior to re-suspending in DMEM wash at 50x10^{6} parasites per mL.

In our *in vitro* studies, the LV39 strain *L. major* parasites that used routinely in our laboratory and the A2 strain *L. major* that was used to generate the gp63KO parasites had very similar growth characteristics and patterns of phagocytosis by macrophages. Therefore, LV39 was used as the WT control for all of the *in vivo* experiments. The advantages to this approach were that we have used the LV39 strain for decades and have extensive experience and familiarity with all aspects of its growth and development in culture and *in vivo*, as well allowing us to utilize a common control for all experiments,

thus allowing comparison between all of the separate experiments performed. It was felt that these advantages outweighed the disadvantages of utilizing separate controls for the LPGKO and gp63KO parasites, given the similar performance of these two strains in our initial *in vitro* experiments.

Gp63KO parasites were developed from the *L. major* strain NIH S (MHOM/SN/74/Seidman) clone A2 parasite as described in Joshi et al. (1998, 2002). LPGKO parasites were developed from the *L. major* strain LV39 (MRHO/Sv/59/P) Clone 5 parasite as described in Späth et al. (2003). Cultures of these parasites were routinely checked for lack of gp63 or LPG expression by Western blot analysis of parasite lysates.

Parasite Quantification

Parasite burden was evaluated using a limiting dilution assay (Lima et al., 1997). Results were scored and the results evaluated using the ELIDA program (ELIDA Software© 1985-2005, Carl Taswell) for statistical analysis (Taswell, 1987).

Cytokine Assays

Popliteal and inguinal lymph nodes draining the infected footpads were removed from euthanized mice. Lymph nodes from multiple mice in the same treatment groups were pooled. Lymph nodes were placed in DMEM and squashed using sterile metal screens to release cells. Mixed cell populations isolated from the lymph nodes were washed and pelleted 3 times in DMEM at 1000 rpm. Live cells were diluted to 5 x 10^6 cells/ml in DMEM cell media [DMEM, as described above, but containing 0.5% normal mouse serum] and added to 24 well plates at 1mL/well. *L. major* promastigotes were added to the plates at 2 x 10^6 parasites/well. Lymph node cells were cultured at 37 °C for

48 h at which time supernatants were collected and stored at -20 °C. Commercially available ELISA's were performed to determine levels of IFN-γ, IL-12, and IL-4 (BD Pharmingen, San Jose, CA) in supernatants. ELISA assays were performed in triplicate.

Statistical analysis

The Students paired *t* test, analysis of variance (ANOVA) & repeated measures general linear model (GLM) were used for statistical analyses of the data (Systat 8.0 & SPSS 16.0 (Systat Software, Inc., Chicago, IL & SPSS, Inc., Chicago, IL). The parasite numbers per footpad was analyzed using the ELIDA software (Taswell, 1987) for initial analysis and, secondarily, ANOVA or GLM on linear plots of the data. Differences were considered significant when $p < 0.05$.

RESULTS

Footpad lesion development in C3KO mice infected with wild type (WT) Leishmania major

Footpad injection of mice with WT *L. major* promastigotes resulted in the appearance of measurable lesions. Infection of C3KO mice with WT *L. major* resulted in the development of lesions that were significantly larger than those in the resistant control mice (Figure 1.1). Initial lesion development was equivalent to controls or, in some of the replicate experiments (data not shown), delayed slightly for the C3KO mice at early timepoints, but lesion size then equaled or surpassed that of the resistant strain. Lesion persistence was also increased for the C3KO strain compared to resistant C57BL/6 control mice, with lesion persistence up to 60 days after lesions were fully resolved for the resistant mice. Similar results were seen in C3KO mice infected with $1x10^5$ WT *L. major* (data not shown). Using a repeated measures GLM, C3KO infected animals had lesions that developed in a significantly ($p<0.05$) different pattern from the resistant controls.

WT L. major parasite numbers in infected C3KO mice

C3KO mice had significantly ($p < 0.05$ from ELIDA (Taswell, 1987)) greater parasite numbers/footpad than in the resistant controls by day 21 and onwards through day 80 (Figure 1.2). Initially higher levels of parasites were detected at day 8, with progression to lower levels at day 14 (Figure 1.2) in the C3KO mice compared to resistant C57BL/6 controls, but this progressed to higher numbers than seen in controls and the parasite numbers persisted for a much longer period of time in the C3KO animals, compared to resistant controls. The progression of parasite replication (Figure

1.2) within the footpad paralleled that of the footpad lesion development. C3KO infected animals had parasite numbers that developed in a significantly ($p<0.05$) different pattern from the resistant controls, but the infection was eventually controlled.

Cytokine responses in WT L. major-infected C3KO mice

Cytokine assays of WT *L. major* infected C3KO mice showed significant differences ($p < 0.05$) in IFN-γ and IL-12 levels at various timepoints compared to resistant controls (Figures 1.3A and 1.3B). The cytokine pattern for IFN-γ production paralleled the pattern of response of both the footpad lesions and the parasite numbers, in that it was initially equal to or lower than resistant animals, then higher as the experiment progressed, being prolonged in duration compared to the cytokine response of resistant controls. IL-12 was almost always decreased compared to controls.

Footpad Lesion Development in C3KO and C57BL/6 mice infected with gp63KO L. major

Footpad injection of mice with both gp63KO and WT *L. major* promastigotes resulted in the appearance of measurable lesions. Infection of resistant control mice (C57BL/6) with gp63KO *L. major* resulted in the development of lesions that were significantly different ($p<0.05$) from those from C57BL/6 mice infected with WT *L. major*, persisting for a longer duration with a slower development of onset (Figure 1.4). The lesions in the gp63KO-infected C57BL/6 group paralleled those seen in the C3KO mice infected with WT *L. major*, with no significant differences seen between these two groups at individual timepoints ($p>0.05$). The C3KO mice infected with gp63KO *L. major* had a greatly expanded time-course of both development and resolution compared to any of the other groups ($p<0.05$), although maximal lesion size was not appreciably

different. Using repeated measures GLM, gp63KO-infected groups had lesions that developed in a significantly ($p<0.05$) different pattern from their WT-infected controls. However, when analyzed by GLM for significant overall differences, there was no difference between the gp63KO-infected C57BL/6 groups and the WT-infected C3KO groups.

L. major parasite numbers in C3KO and C57BL/6 mice infected with gp63KO L. major

Footpad injection of mice with both gp63KO and WT *L. major* promastigotes resulted in parasite infection, replication and eventual clearance in both C57BL/6 and C3KO mice. Parasite numbers persisted for a significantly longer period of time in the gp63KO-infected animals, compared to WT parasite infections. C57BL/6 mice infected with gp63KO *L. major* had increased numbers of parasites/footpad from day 28 through day 80 compared to controls (C57BL/6 infected with WT *L. major*). These C57BL/6 mice infected with gp63KO parasites had parasite numbers that paralleled those of the C3KO mice + WT parasites, but at a slightly lower level of parasites/footpad (although not significantly lower). C3KO mice infected with gp63KO parasites had significantly ($p < 0.05$ from ELIDA (Taswell, 1987)) greater parasite numbers/footpad than in C3KO control mice infected with WT parasites by day 50 and onwards, with greatly increased persistence of high numbers of parasites in the C3KO mice infected with gp63KO parasites (Figure 1.5). C3KO mice infected with gp63KO *L. major* had initially decreased parasite burden with a significantly increased magnitude and longevity of parasite burden over time (Figure 1.5). The progression of parasite replication within the footpad paralleled that of the footpad lesion development.

Cytokine responses in gp63KO L. major-infected C3KO mice

Cytokine assays of gp63KO *L. major* infected C57BL/6 and C3KO mice showed significant differences ($p < 0.05$) in IFN-γ and IL-12 levels at various timepoints compared to resistant controls (Figures 1.6A and 1.6B). The cytokine levels for IFN-γ in gp63KO-infected mice were initially lower than WT-infected control animals on days 8 and 14, with no essential differences seen between gp63KO-infected and WT-infected mice of the same strain at later timepoints. IL-12 was almost always decreased in gp63KO-infected mice compared to WT-infected controls (barring day 50 C57BL/6 mice).

Footpad Lesion Development in BALB/c, C3KO and C57BL/6 mice infected with LPGKO L. major

Footpad injection of mice with LPGKO *L. major* did not result in the appearance of significant or measurable lesions, regardless of the resistant or susceptible phenotype of the mice (Figure 1.7). All of the mice strains, including C3KO mice, injected with LPGKO parasites exhibited the same non-progression of lesions. These results corroborate those of Späth et al. (2003) in that the parasites produce a long-lasting infection without evidence of pathology.

L. major parasite numbers in BALB/c, C3KO and C57BL/6 mice infected with LPGKO L. major

Footpad injection of mice with LPGKO *L. major* did not result in a normal progression or level of magnitude of parasite burden in the affected mouse, regardless of the resistant or susceptible phenotype of the mice All of the mice strains injected with

LPGKO parasites exhibited the same low-level parasite burden without significant progression or change over time (Figure 1.8). These results corroborated those of Späth et al. (2003) in that the parasites produce a long-lasting infection without evidence of pathology. Of interest is the fact that there is a 10-1000 fold difference in the numbers of parasites seen in the C3KO animals compared to the resistant controls in the later stages of the infection (days 60 and 142) (Figure 1.8). Also of interest is the continued slight increase in the number of parasites in the C3KO and BALB/c mice over time. However, this may be an artifact of sampling, in that the specific animals sampled at these times may have had a slightly greater infection than others at those specific times.

Cytokine Response in BALB/c, C3KO and C57BL/6 mice infected with LPGKO L. major

Footpad injection of mice with LPGKO *L. major* did not result in significant or measurable cytokine response upon restimulation of lymph node cultures, regardless of the resistant or susceptible phenotype of the mice. All of the mouse strains infected with LPGKO parasites exhibit the same non-response to restimulation of all cytokines assayed (IL-12, IFN-γ, IL-4) (Figures 1.9A-C). All of the LPGKO-infected mice at all of the timepoints had cytokine assay values at or below the limit of detection by the ELISA kits.

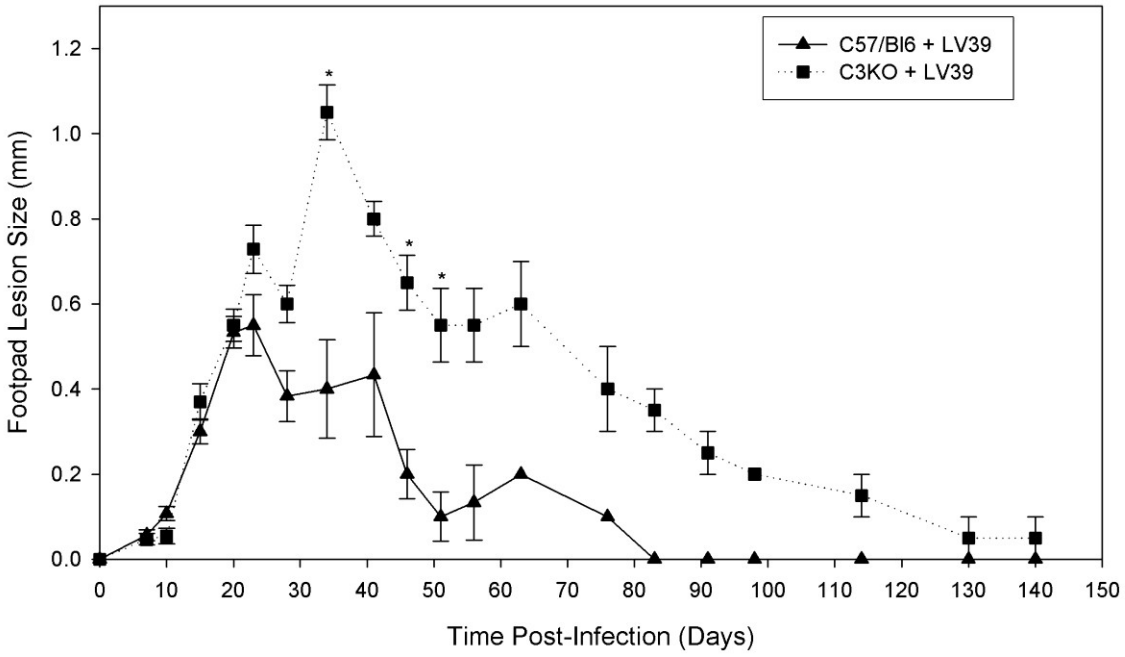

Figure 1.1: C3KO and resistant control mice footpad lesion development over time in animals infected with 1 x 10^6 wild type (WT) *L. major* promastigotes. Error bars reflect standard deviations. Significant differences ($p < 0.05$) at individual timepoints between C3KO and resistant mice are indicated by an *. Overall plots are significantly different ($p<0.05$) over time, using GLM with repeated measures. Data shown is from a representative experiment of three performed (n=25/group).

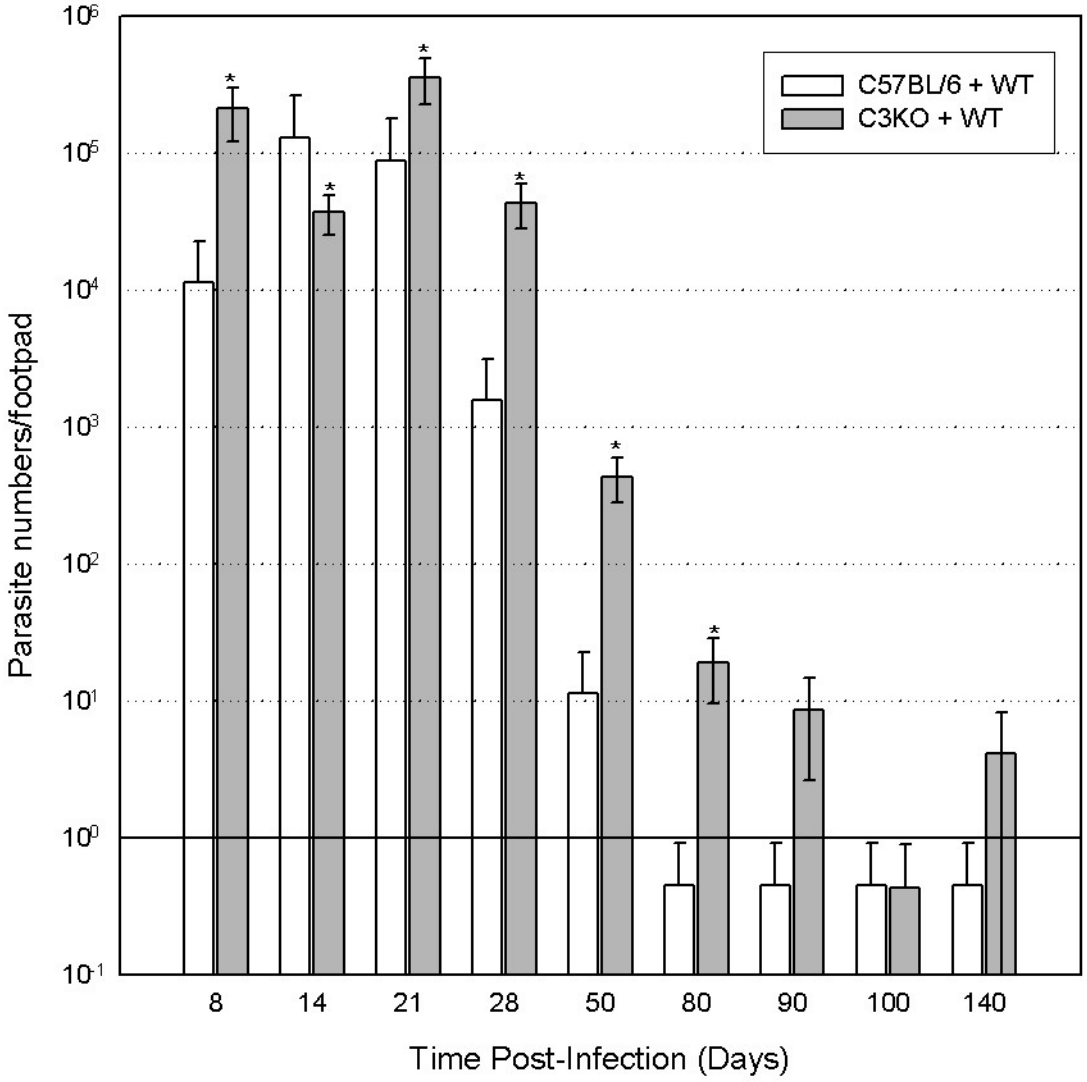

Figure 1.2: Analysis of parasite burden in footpad lesions from C3KO and resistant C57BL/6 mice infected with $1x10^6$ WT *L. major* promastigotes. Error bars reflect 95% confidence intervals. Significant differences ($p < 0.05$) are indicated by an *. Each timepoint group consists of three to four mice, and data shown is from a representative experiment of three performed.

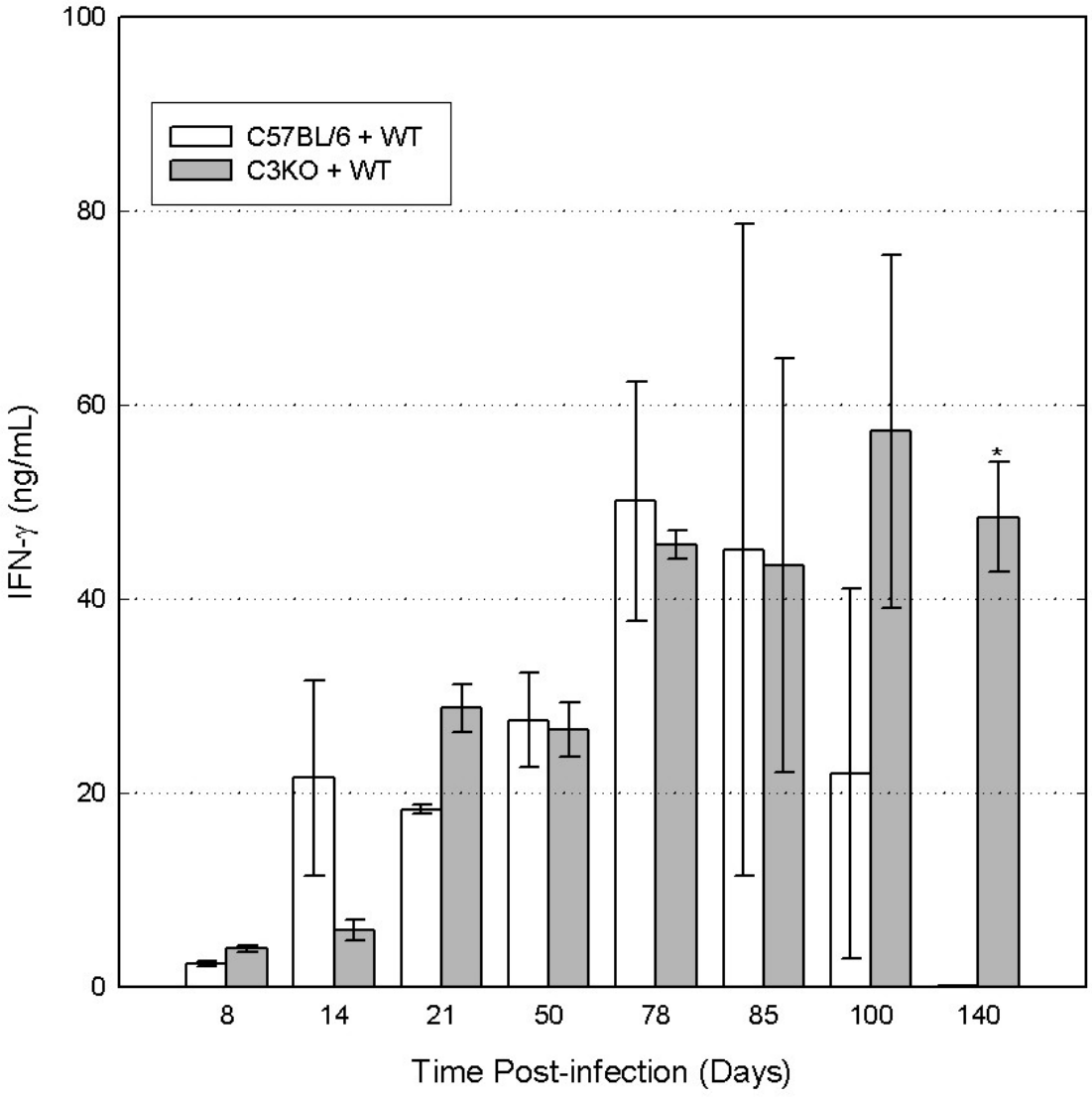

Figure 1.3A: IFN-γ assay results for C3KO mice infected with 1 x 10^6 WT *L. major* promastigotes compared to resistant strain (C57BL/6). Error bars reflect standard deviations. Significant differences ($p < 0.05$) are indicated by an *. Each timepoint group consists of three to four mice, and data shown is from a representative experiment of three performed.

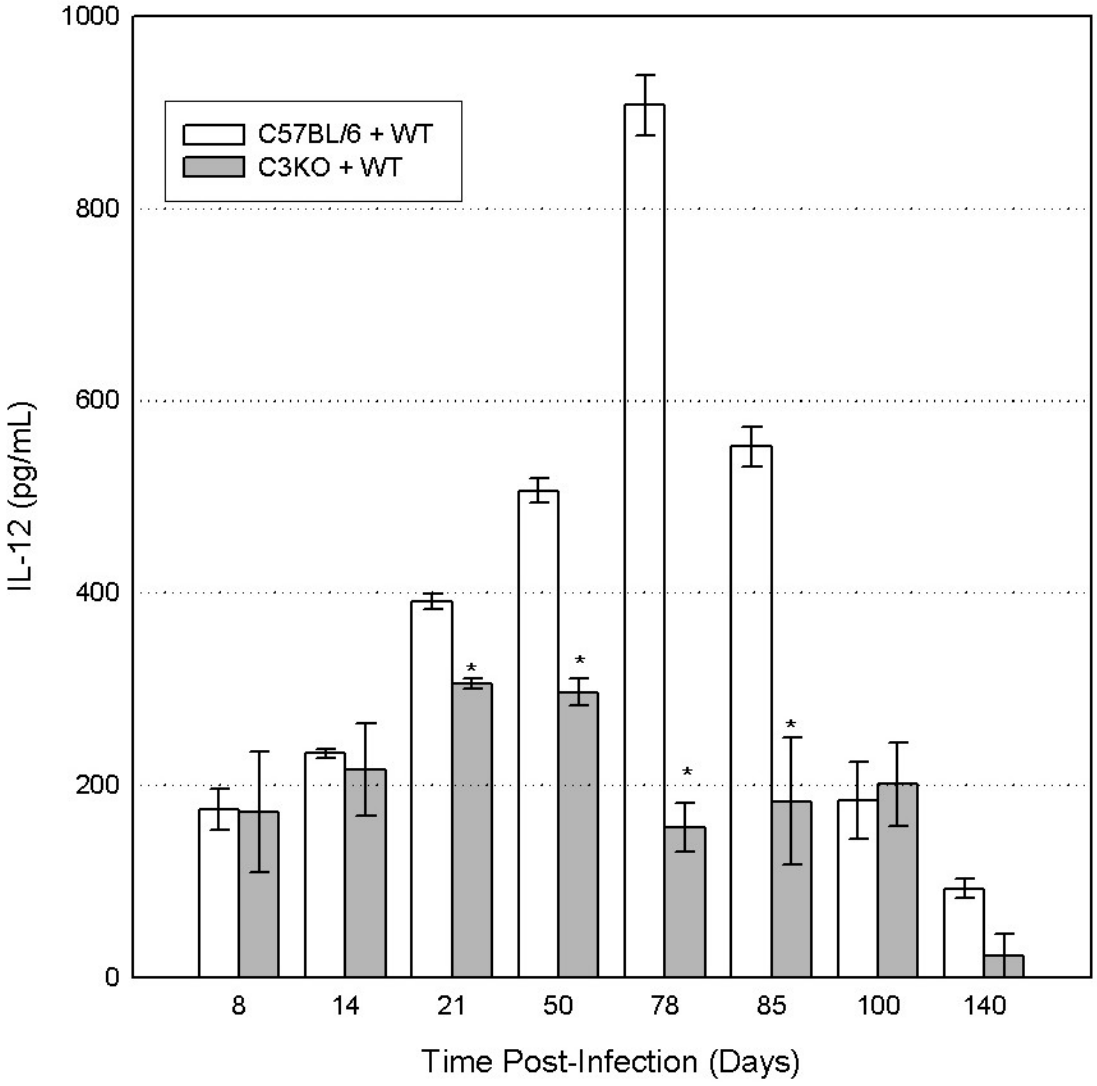

Figure 1.3B: IL-12 assay results for C3KO mice infected with 1 x 10^6 WT *L. major* promastigotes compared to resistant strain (C57BL/6). Error bars reflect standard deviations. Significant differences ($p < 0.05$) are indicated by an *. Each timepoint group consists of three to four mice, and data shown is from a representative experiment of three performed.

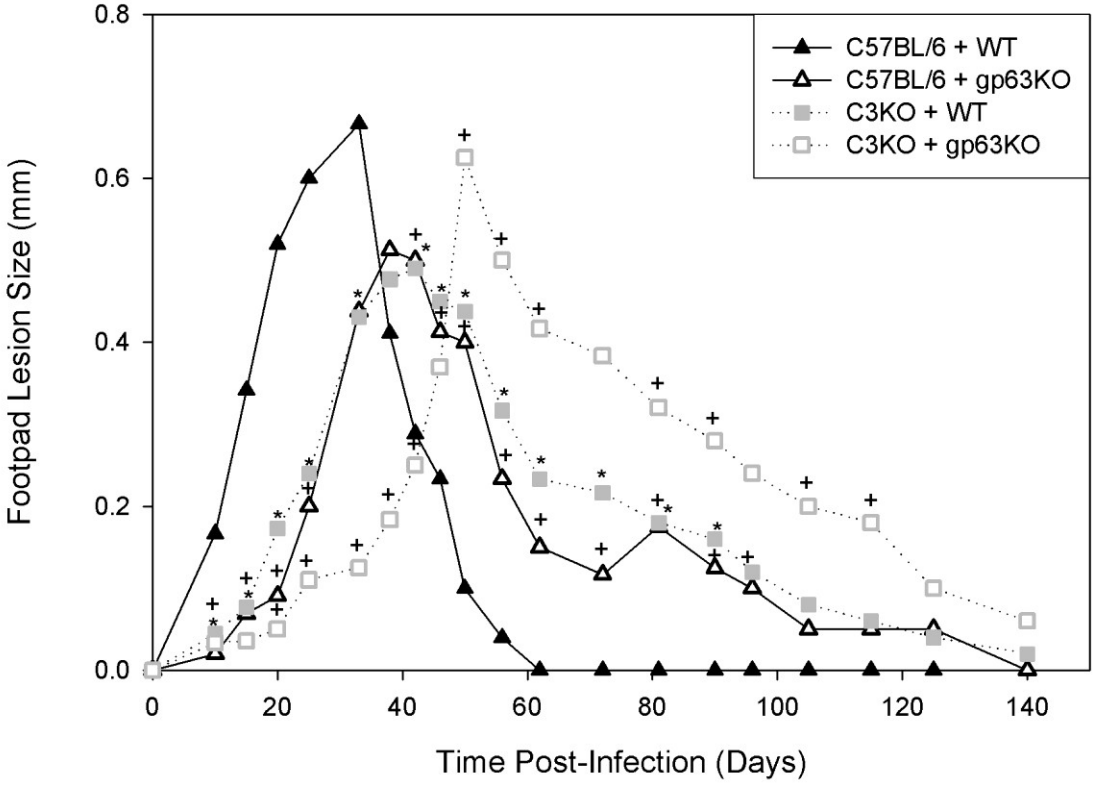

Figure 1.4: C3KO and C57BL/6 (resistant) mice footpad lesion development over time when infected with 1 x 10^6 WT or gp63KO *L. major* promastigotes. Error bars are not included to facilitate visualization of the data. When WT-infected C3KO (■) and C57BL/6 (▲) groups were compared at individual timepoints, significant differences ($p < 0.05$) are indicated by an *. Likewise, when gp63KO-infected (□ and Δ) and WT-infected (■ and ▲) groups were compared at individual timepoints, significant differences ($p < 0.05$) are indicated by a +. When the groups were analyzed by GLM for significant ($p< 0.05$) overall differences, the groups that differed were the WT-infected C57BL/6 and C3KO groups, the C57BL/6 WT-infected and gp63KO-infected groups, and the C3KO WT-infected and gp63KO infected groups. There was no significant difference between the C57BL/6 + gp63KO and the C3KO + WT groups using GLM analysis. Data shown is from a representative experiment of three performed (n=25/group).

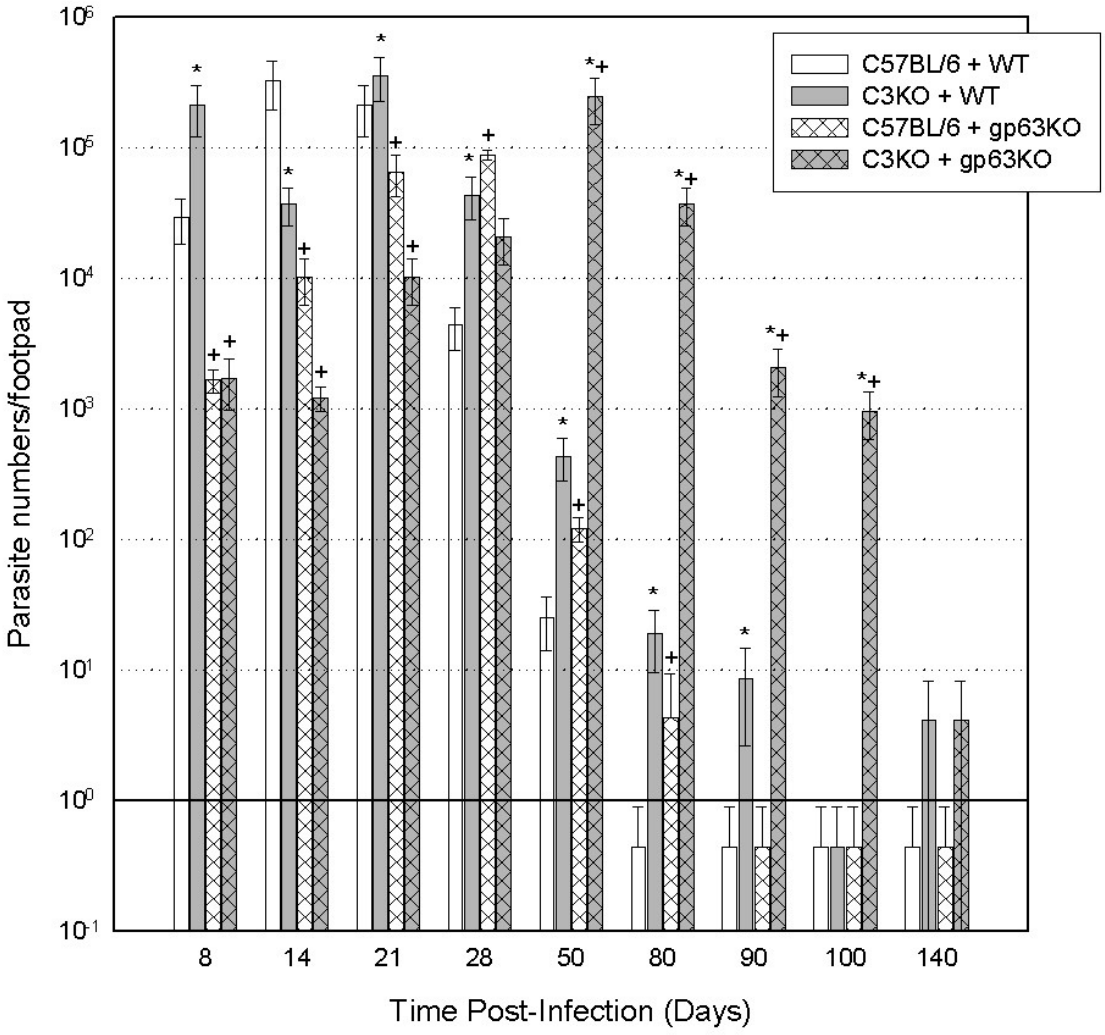

Figure 1.5: C3KO and C57BL/6 (resistant) mice parasite numbers/footpad over time when infected with 1 x 10^6 WT or gp63KO *L. major* promastigotes. Error bars reflect 95% confidence intervals. Significant differences ($p < 0.05$) at individual time points between C3KO and resistant mice are indicated by an *. Significant differences between gp63KO-infected and WT-infected mice of the same strain are indicated by a +. Each timepoint group consists of three to four mice, and data shown is from a representative experiment of three performed.

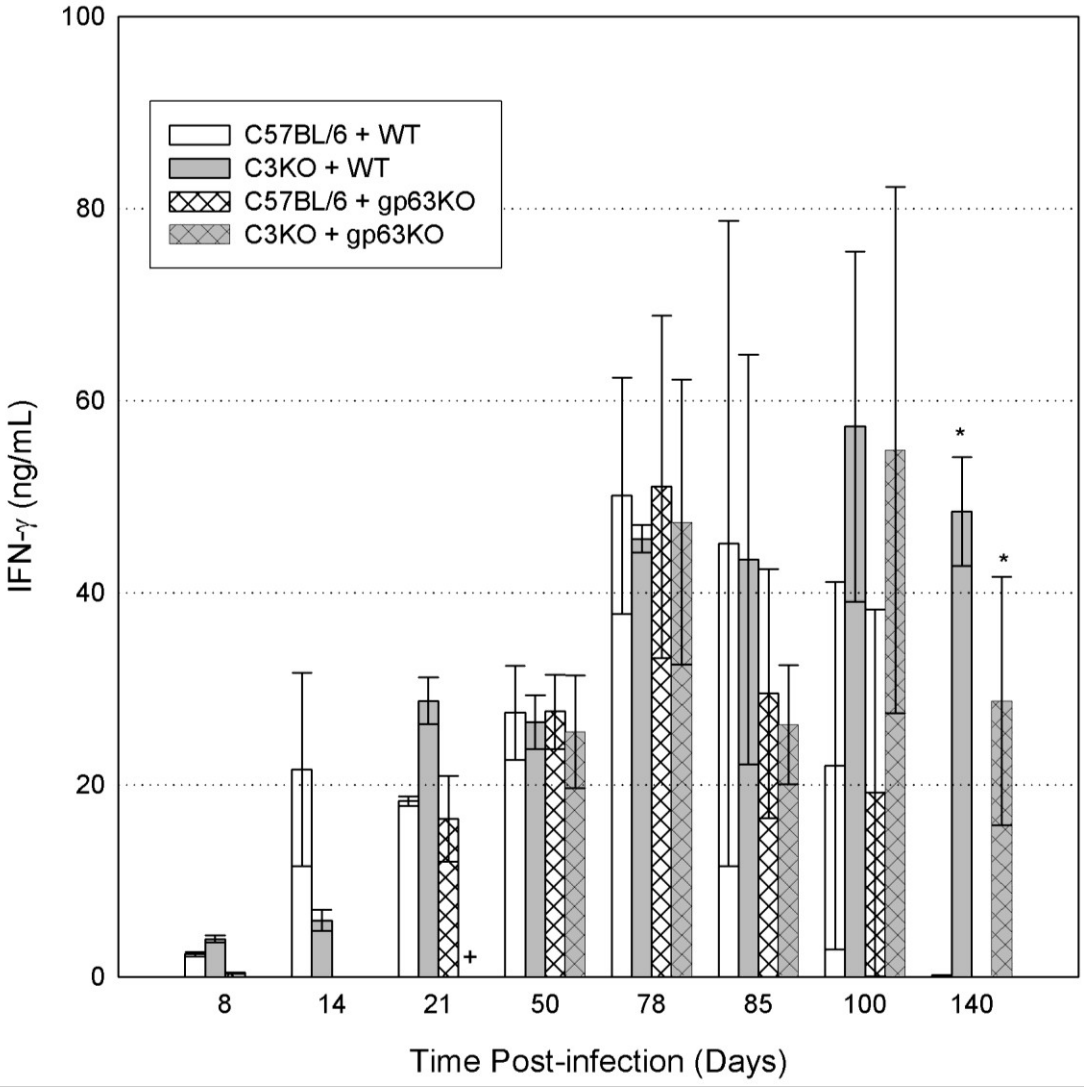

Figure 1.6A: IFN-γ assay results for C3KO and C57BL/6 mice infected with 1 x 10^6 gp63KO *L. major* promastigotes compared to C3KO and C57BL/6 mice infected with 1 x 10^6 WT *L. major* promastigotes. Error bars reflect standard deviations. Significant differences ($p < 0.05$) at individual time points between C3KO and resistant mice are indicated by an *. Significant differences between gp63KO-infected and WT-infected mice of the same strain are indicated by a +. Each timepoint group consists of three to four mice, and data shown is from a representative experiment of three performed.

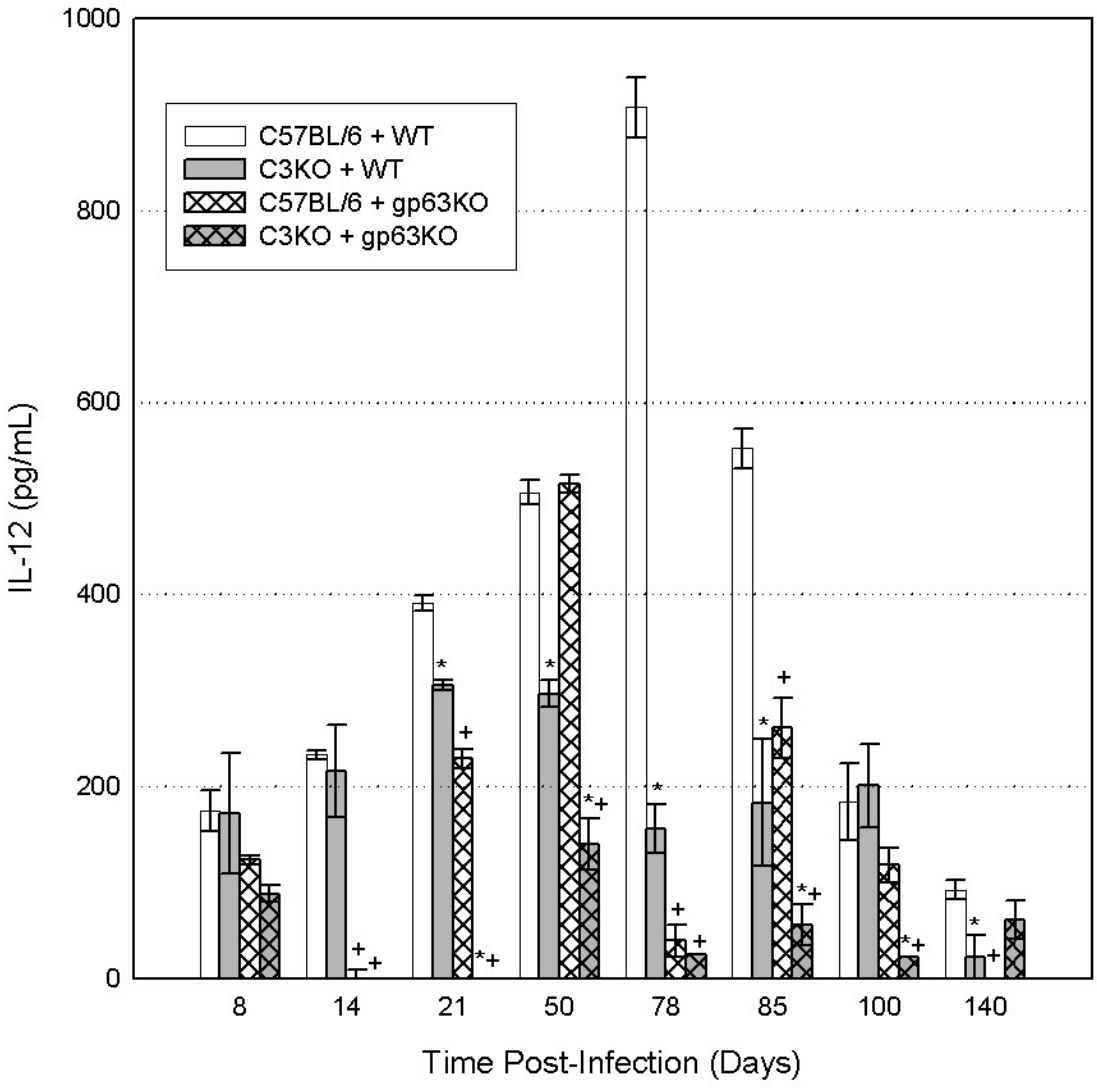

Figure 1.6B: IL-12 assay results for C3KO and C57BL/6 mice infected with 1 x 10^6 gp63KO *L. major* promastigotes compared to C3KO and C57BL/6 mice infected with 1 x 10^6 WT *L. major* promastigotes. Error bars reflect standard deviations. Significant differences ($p < 0.05$) at individual time points between C3KO and resistant mice are indicated by an *. Significant differences between gp63KO-infected and WT-infected mice of the same strain are indicated by a +. Each timepoint group consists of three to four mice, and data shown is from a representative experiment of three performed.

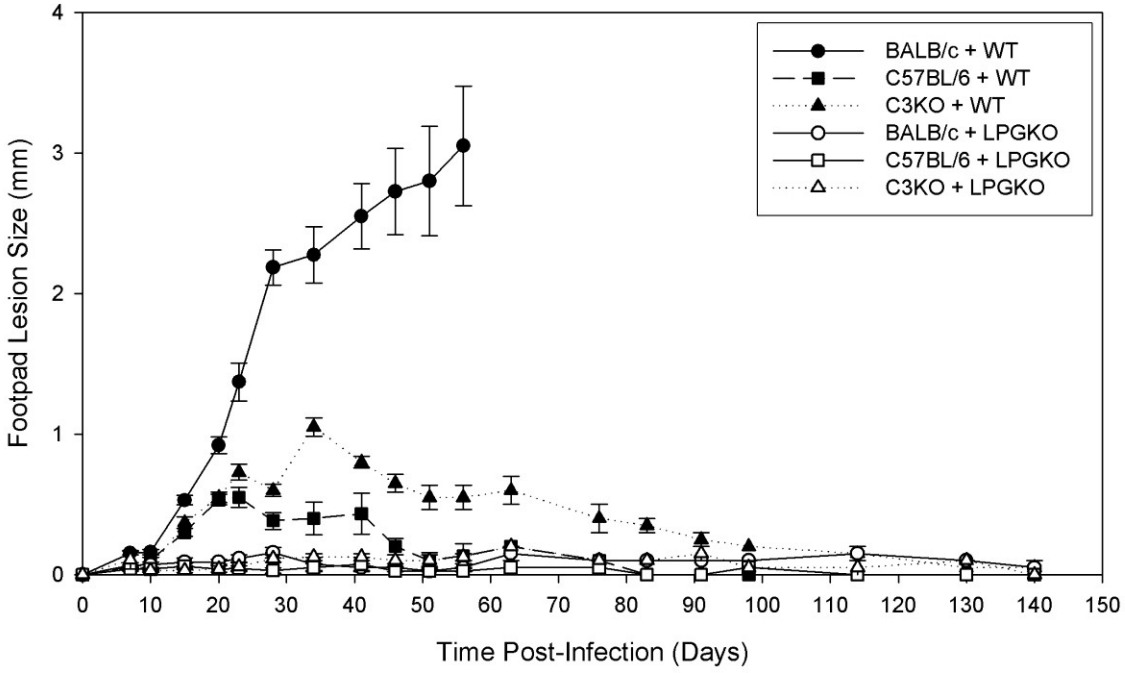

Figure 1.7: C3KO, BALB/c (susceptible) and C57BL/6 (resistant) mice footpad lesion development over time in animals infected with either 1 x 10^6 WT or 3 x 10^6 LPGKO *L. major* promastigotes. Error bars reflect standard deviations. There are no significant differences ($p < 0.05$) between any of the LPGKO treated animals. Data shown is from a representative experiment of two performed (n=18/group).

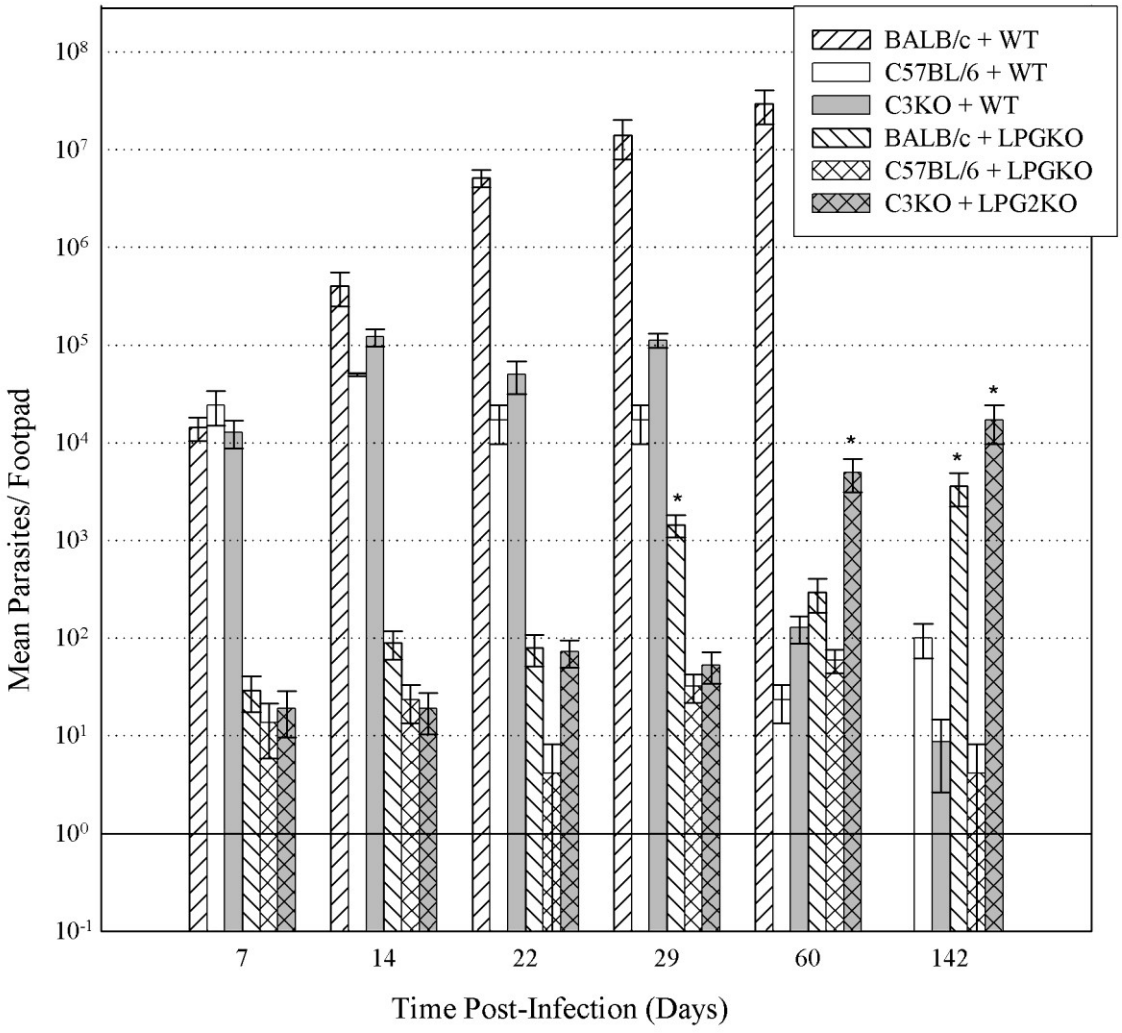

Figure 1.8: C3KO, BALB/c (susceptible) and C57BL/6 (resistant) mice parasite numbers/footpad over time in animals infected with either 1 x 10^6 WT or 3 x 10^6 LPGKO *L. major* promastigotes. Error bars reflect 95% confidence intervals. Significant differences ($p < 0.05$) at individual timepoints between C57BL/6 and other LPGKO-infected mice strains are indicated by an *. Each timepoint group consists of three mice, and data shown is from a representative experiment of two performed.

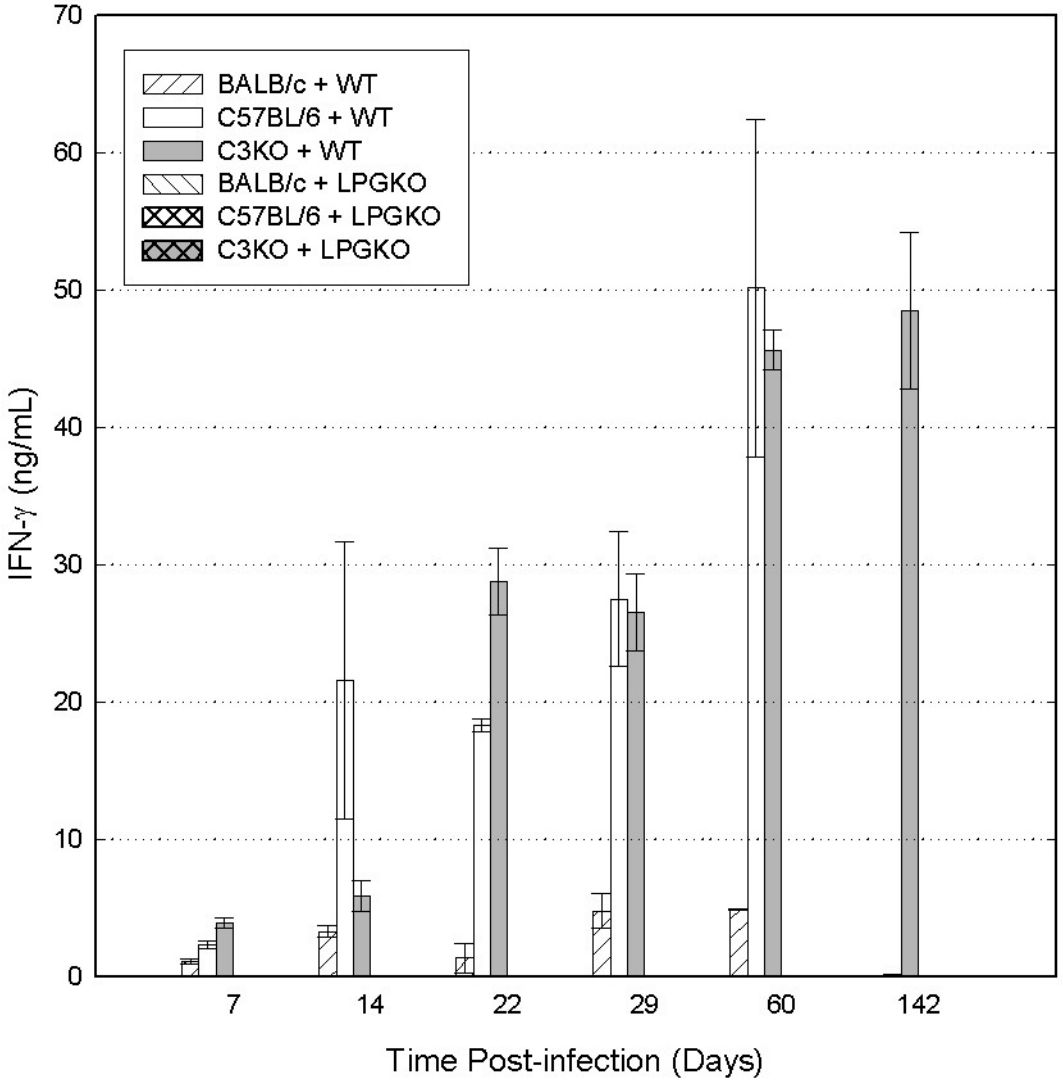

Figure 1.9A: IFN-γ assay results for BALB/c, C3KO and C57BL/6 mice infected with 3 x 10^6 LPGKO *L. major* promastigotes compared to BALB/c, C3KO and C57BL/6 mice infected with 1 x 10^6 WT *L. major* promastigotes. Error bars reflect standard deviations. Mice infected with LPGKO parasites had no cytokine response to infection. The vast majority of differences between LPGKO-infected and WT-infected mice of the same strain were significant ($p<0.05$), except in instances where the cytokine levels of the WT-infected mice approached or were at zero. Each timepoint group consists of three mice, and data shown is from a representative experiment of two performed.

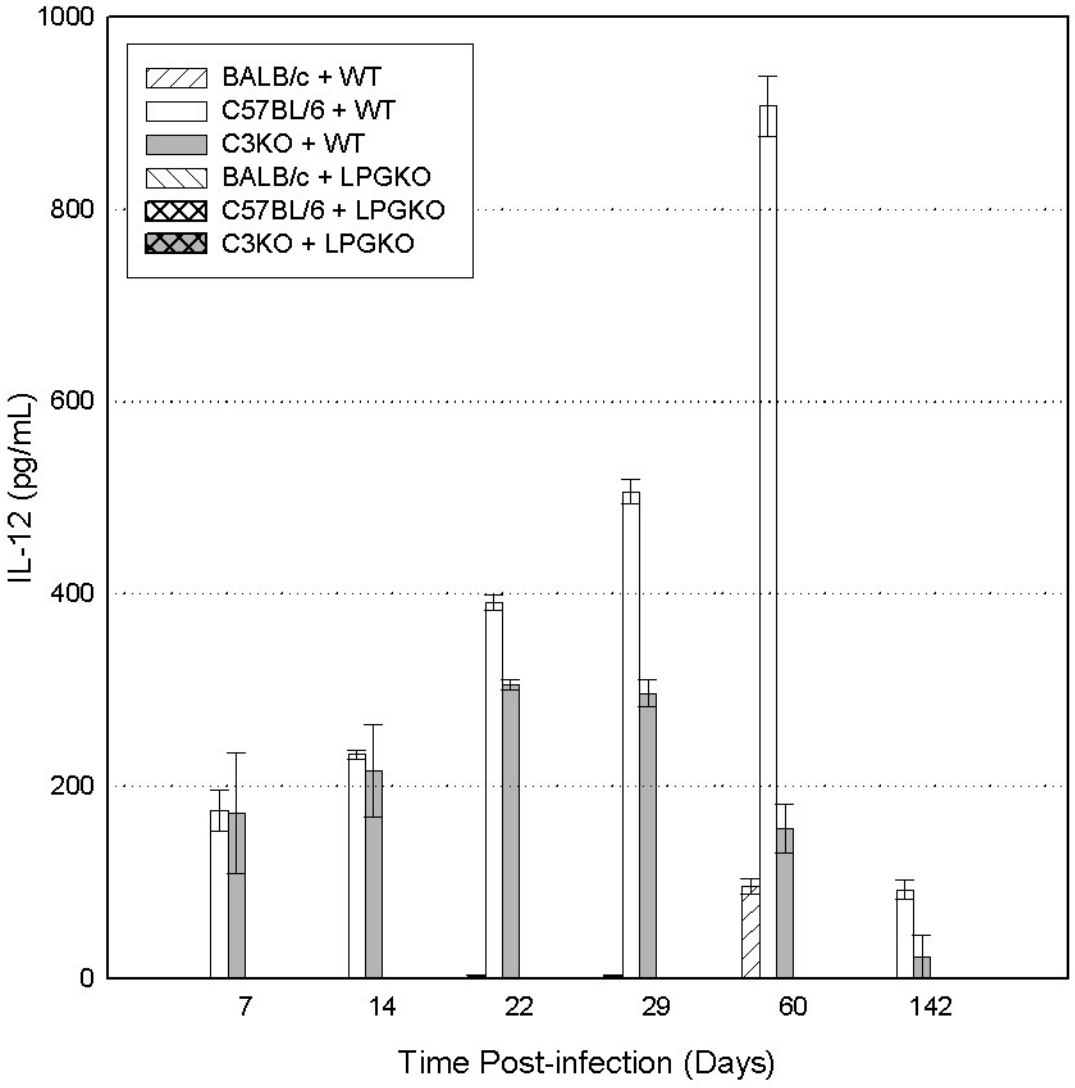

Figure 1.9B: IL-12 assay results for BALB/c, C3KO and C57BL/6 mice infected with 3 x 10^6 LPGKO *L. major* promastigotes compared to BALB/c, C3KO and C57BL/6 mice infected with 1 x 10^6 WT *L. major* promastigotes. Error bars reflect standard deviations. Mice infected with LPGKO parasites had no cytokine response to infection. The vast majority of differences between LPGKO-infected and WT-infected mice of the same strain were significant ($p<0.05$), except in instances where the cytokine levels of the WT-infected mice approached or were at zero. Each timepoint group consists of three mice, and data shown is from a representative experiment of two performed.

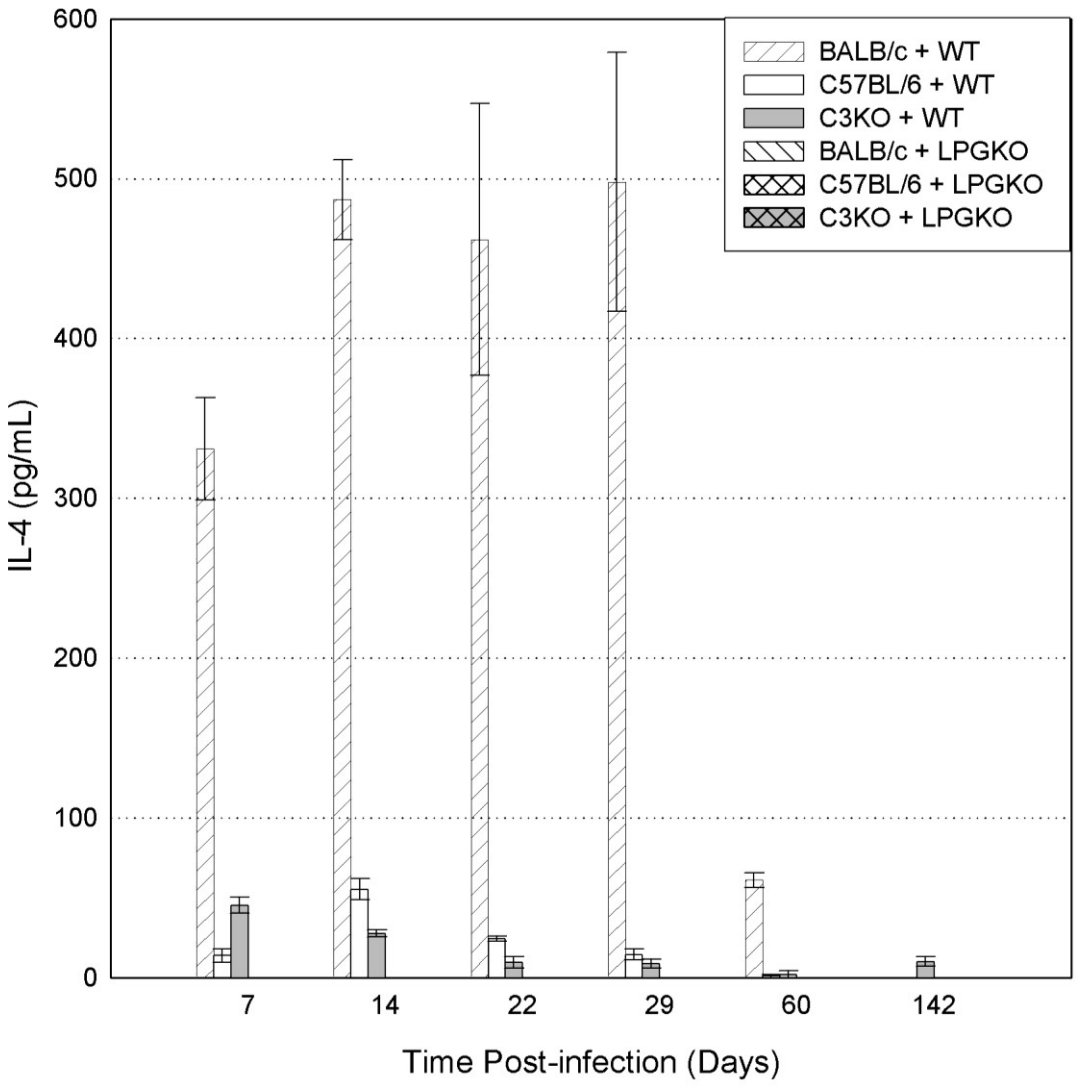

Figure 1.9C: IL-4 assay results for BALB/c, C3KO and C57BL/6 mice infected with 3 x 10^6 LPGKO *L. major* promastigotes compared to BALB/c, C3KO and C57BL/6 mice infected with 1 x 10^6 WT *L. major* promastigotes. Error bars reflect standard deviations. Mice infected with LPGKO parasites had no cytokine response to infection. The vast majority of differences between LPGKO-infected and WT-infected mice of the same strain were significant ($p<0.05$), except in instances where the cytokine levels of the WT-infected mice approached or were at zero. Each timepoint group consists of three mice, and data shown is from a representative experiment of two performed.

DISCUSSION

The role of C3 in the development of disease pathology has been poorly understood to date. Some evidence has suggested that complement may be necessary for opsonization of the *L. major* parasite (Dominguez and Torano, 1999) and eventual facilitation of *L. major* phagocytosis and disease progression, particularly in humans (Dominguez et al., 2002; Dominguez et al., 2003). Similar evidence has suggested that there is no significant lesion development seen in transgenic mice undergoing constant C3 consumption due to expression of cobra venom factor, suggesting that C3 is required for normal disease progression (Jacobs et al., 2005). Other evidence has suggested that complement is a key component in destruction of *Leishmania* (Moreno et al., 2007), with lack of complement being equated with enhanced disease pathology and increased numbers of parasites surviving initial infection. Our work has demonstrated that mice lacking C3 exhibit a different temporal disease course than that seen in resistant mice but do eventually develop and clear disease.

The eventual development and clearance of the lesions suggests that C3 and opsonization are not essential to *L. major* phagocytosis and infection, but that they alter the host response to the parasite. If C3 was essential for phagocytosis and progression of *L. major* infection, then infection of C3KO mice with *L. major* would result in no disease progression, no lesion development, and certainly no increases in parasite numbers over time. However, it is obvious that C3 plays a role in normal *L. major* infection, as C3KO animals have noticeable changes in the progression of not only lesions, but also parasite amplification and cytokine responses to *L. major*. The role of C3 may be one of opsonization, allowing increased and more efficient phagocytosis of *L. major*, but

without being the sole ligand for stimulation of phagocytosis of *L. major*. Thus the initially reduced parasite burden and lesions, which would indicate that the histiocytic response was reduced and that *L. major* phagocytosis (and therefore protection) was reduced in early infection. Presumably this was due to the lack of C3 opsonization and resultant enhancement of phagocytosis, as this is seen in human *in vitro* experiments (Dominguez and Torano, 1999). However, as there are other routes by which *L. major* may bind to the phagocytic cells and induce phagocytosis (Mosser and Edelson, 1985; Wilson and Pearson, 1988; Bosetto and Giorgio, 2007; Tuon et al., 2008), there is an eventual increase in parasite burden and lesion size over and above that of the controls. Along with this is the observation that C3 must be involved not only in phagocytosis of *L. major*, but also in determining survival and the long-term immune response to the parasite. This is born out by the increased time of infection seen in C3KO animals, suggesting that lack of C3 results in not only inefficient phagocytosis, but also inefficient clearance of parasites. This increased clearance time for *L. major* infection suggests that C3 opsonization and the development of a Th1 response are linked to some degree, with C3 opsonization of *L. major* leading to increased (normal) resistance and development of a Th1 response with rapid parasite clearance. Similar conclusions may be drawn from the cytokine responses of C3KO mice, which lag behind those of resistant mice (Figures 1.3 and 1.6), suggesting that there is a decreased ability of the immune system to recognize and respond to the parasite. However, the cytokine responses remain those of a Th1 response and do not switch to a Th2-type response, suggesting that C3 is not necessary for development of a protective Th1 response in otherwise resistant mice.

The role of gp63 in the *in vivo* response to *L. major* is similar to that of C3, with infections by gp63KO parasites causing decreased clearance of the parasite and increased time for both development and resolution of the infection compared to mice of the same strain (C3KO or C57BL/6) infected with WT *L. major*, based on lesion measurements (Figure 1.4) and parasite numbers (Figure 1.5). Resistant mice (C57BL/6) infected with gp63KO parasites had very similar courses of disease progression to C3KO mice infected with WT *L. major* (Figures 1.4 and 1.5). This similarity suggests that both gp63 and C3 are equivalently important for the development of the normal *L. major* infection, since without either component, there is a similar change in the progression of lesion development and parasite numbers (Figures 1.4 and 1.5). It may be that there is an *in vivo* interaction between gp63 and C3 that produces this similar progression of disease, perhaps due to enhancement of phagocytosis and thus immediate protection of *L. major* or due to enhanced host immune response to the parasites. However, the increased time of lesion development and resolution seen in C3KO animals infected with gp63KO *L. major* suggests that gp63 may be more than just an important molecule for interactions of *Leishmania* with complement. Otherwise, lesion size and parasite numbers would be equal between gp63KO-infected C3KO and WT-infected C3KO groups (Figures 1.4 and 1.5). This is already known, to some degree, as gp63 is known to interact with both complement receptor 1 and complement receptor 3 (Mosser and Brittingham, 1997; Brittingham et al., 1999) during *L. major* phagocytosis, although its action has not been clearly separated from the C3 opsonin in any of these experiments. It would appear that gp63 interaction with C3 is important for initial interaction of the parasite with phagocytic receptors and normal progression of infection. It is not a requisite for

interaction between *L. major* and the host, and infection and disease progression, occurs, albeit at a reduced rate. The mechanism for this is unclear, but the lack of gp63 may affect the murine host in an equivalent manner to the lack of C3, affecting opsonization and clearance of the parasites in a temporal manner.

Unlike gp63 or C3, LPG appears to be required by the parasite for development of a normal infection in the mouse. In any of the three strains of mice used in our experiments, LPGKO parasites caused little to no lesion development and only limited low-level parasite replication in the host, even after almost 5 months of infection. This is similar to the findings published by Späth et al. (2003) utilizing the same strain of parasites, which showed that LPGKO parasites caused a persistent low-level infection with no appreciable lesion development. There was a limited increase in parasite numbers (parasite replication) in mice lacking C3, compared to resistant or susceptible controls, which may be due to the lack of C3-driven parasite opsonization and/or complement-mediated parasite destruction. LPG has been recognized as an independent ligand of TLR2 (Becker et al., 2003; de Veer et al., 2003) and thus may be involved in and acting via a completely separate pathway than that of the complement cascade and opsonization. LPG serves as a known binding site for C3 (Talamas-Rohana et al., 1990), but, as we have shown here (Figures 1.7, 1.8, 1.9), has very different effects on host-parasite interactions than those produced by the presence or absence of C3. It may be that the lack of LPG is such a significant detriment to the parasite that the comparatively minor effects of the lack of interaction with C3 and associated complement receptors are un-noticed or cannot be readily identified.

The temporal lag in response of the C3 KO mice to *L. major* infection indicates that C3 opsonization is one of the more significant routes by which the parasite is recognized and phagocytosed by macrophages and possibly one of the mechanisms that induces the host's innate/adaptive immune response. However, since there is still a response to the parasite in the C3KO mice, opsonization is not required for parasite recognition and host response. Since C3 opsonization is not required, other ligands and receptors therefore must be utilized by host macrophages in recognition and phagocytosis of *L. major*. Among these receptors would be the scavenger receptor, mannose-fucose receptor, Fc-γ receptor, and fibronectin receptor, as well as TLRs. These receptors could be recognizing ligands other than C3b and iC3b, including *L. major* LPG and/or gp63, although it is apparent that the lack of gp63 does not prevent parasite survival and host disease. The more precise effects and interactions of host receptors, gp63 and LPG would be best examined *in vitro*.

CHAPTER 2

THE ROLE OF COMPLEMENT RECEPTORS AND THE *LEISHMANIA MAJOR* SURFACE MOLECULES LIPOPHOSPHOGLYCAN AND GP63 ON *IN VITRO* MACROPHAGE RECOGNITION OF *LEISHMANIA MAJOR*

Authors
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CHAPTER SYNOPSIS

Leishmania major is phagocytosed by macrophages and replicates within the phagolysosome. Recognition and phagocytosis of *L. major* is regulated by interactions of parasite surface ligands with macrophage cell surface receptors. Activation of the complement receptors (CR1/2 & CR3) may influence the phagocytosis of and subsequent immune response to the parasite. We utilized peritoneal-derived macrophages or bone marrow-derived macrophages from C57/Bl6 mice and blocked complement receptors CR1/2 (CD21) and CR3. The blocked macrophages were subsequently infected with LV39 wild-type (WT) strain, A2 WT strain, lipophosphoglycan (LPGKO) knockout, and gp63 metalloprotease (gp63KO) knockout *L. major*. Phagocytosis of *L. major* was assessed at 24 hours. Supernatants from macrophage cultures infected for 48 and 72 hours were assayed for monokines (IL-10, IL-6, IL-12, TNF-α, NO). CD4+ T cells from C57/Bl6 mice were added and co-cultured for 5-7 days and the resultant supernatants assayed for Th1/Th2 cytokines (IL-4, IL-12, IFN-γ, IL-6, IL-10, TNF-α, NO). In monokine experiments, IL-6, IL-10, IL-12 and TNF-α were all significantly elevated in preparations from macrophages blocked by CR1/2 and CR3. However, in co-culture experiments, only IL-6 was affected. Despite effects on initial macrophage function and macrophage-produced cytokines, the role of complement receptors in the *in vitro* regulation of adaptive immunity is apparently limited.

INTRODUCTION

Leishmania major promastigotes are phagocytosed by macrophages, dendritic cells, and other phagocytic cells within the mammalian host. Macrophages constitute the primary phagocytic cell population in *Leishmania* infection and serve as a site of immune evasion for the parasite, which can replicate within the phagolysosome. Recognition and initiation of phagocytosis by the macrophage is regulated by membrane receptors, including scavenger receptors, toll-like receptors, mannose-fucose receptors, and the complement receptors. Activation of macrophages and other phagocytic cells by membrane receptors can potentially influence subsequent immune response, both with the innate and adaptive arms of the immune system. *L. major* phagocytosis is enhanced *in vitro* by complement opsonization, suggesting that complement is utilized by the parasite to increase the infection rate of macrophages (Mosser and Edelson, 1985; Puentes et al., 1988; Mosser et al., 1992; Brittingham and Mosser, 1996; Rosenthal et al., 1996).

The complement receptors are essential components of macrophage recognition and phagocytosis. Complement receptors (CR) involved in *Leishmania* infection include the CR1/2 (CD35/CD21) complex and CR3 (CD11b/CD18). CR1/2 recognizes and binds to C3b, as well as possibly *Leishmania* LPG (da Silva et al., 1989; Talamas-Rohana et al., 1990; Rosenthal et al., 1996). CR3 binds to the inactive form of C3b (iC3b), LPG, and possibly gp63 (Mosser and Edelson, 1985; Russell and Wright, 1988; Wilson and Pearson, 1988; Rosenthal et al., 1996). CR3 appears to be primarily involved in phagocytosis of procyclic, low infectivity *Leishmania* (Puentes et al., 1988) and amastigote forms (Guy and Belosevic, 1993; Kelleher et al., 1995), although there is a

reduction in the uptake of *Leishmania* promastigotes when CR3 is blocked (Mosser et al., 1996; Rosenthal et al., 1996). The interaction between CRs and *L. major* has been shown to affect phagocytosis, but may also affect the downstream immune response.

Leishmania surface molecules are important in interaction with the host. The primary structure on the *L. major* cell surface is lipophosphoglycan (LPG). LPG is a long chain glycoconjugate found in abundance on the external membrane of *Leishmania* promastigotes. It is the major cell surface structure on promastigotes, particularly on the infectious metacyclic form, and covers the entire surface of the parasite, including the flagellum (Turco and Descoteaux, 1992). LPG strands are linked to the parasite by a conserved glycosylphosphatidylinositol (GPI) anchor and contain a glycan core with a highly variable repeating saccharide-phosphate region and oligosaccharide cap (Turco, 1990). LPG strands, particularly the apical oligosaccharide cap, are attachment sites for complement, enhancing the interaction of C3b with Factor B (Green et al., 1994; Brittingham and Mosser, 1996). The abundance and importance of LPG as a surface molecule for opsonization by C3 and subsequent CR1 binding to macrophages argues that it may indeed be an important link in the entry of the parasite into macrophages, initiating the innate immune response and subsequently defining the nature of the specific immune response to the parasite.

The most abundant protein on the surface of *Leishmania* promastigotes is a 63 kDa glycoprotein called gp63, or leishmanolysin (Etges et al., 1986). Gp63 expression is increased markedly as promastigotes progress into metacyclogenesis and become more infectious (reviewed in Mosser and Brittingham, 1997). Gp63 is a zinc metalloprotease with a strong ability to degrade a wide variety of proteins, including albumin, casein,

immunoglobulin, hemoglobin, and complement proteins (Chaudhuri et al., 1988). Like LPG, gp63 serves as a C3 acceptor, supporting opsonization by C3 and mediating the attachment of promastigotes to macrophages. It also regulates complement-mediated lysis by proteolytic inactivation of C3b into a form similar to that of inactivated C3b (iC3b) (Brittingham and Mosser, 1996). This iC3b-like form remains opsonic, but does not allow for continuation of the complement cascade. As well as its role as a C3 acceptor, gp63 may also serve as a ligand for attachment of *Leishmania* to macrophages, possibly via the CR3 receptor (Mosser and Brittingham, 1997; Brittingham et al., 1999). This enhancement of binding and use of alternate receptors may affect the interactions between *L. major* and macrophages and thus the resulting nature of the specific immune response to the parasite.

We utilized starch-induced peritoneal macrophages infected with 3 separate strains of *L. major* (LV39, gp63KO, and LPGKO) & co-cultured with CD4+ T cells to investigate the role of parasite surface molecules in induction of monokine & cytokine responses to *L. major* infection. Complement receptor blocking antibodies were used to address the role of macrophage complement receptors in recognition of and response to *L. major*. Bone marrow-derived macrophages infected with the same strains, in conjunction with complement receptor blocking antibodies, were used to investigate the role of parasite surface molecules and macrophage complement receptors on phagocytosis of *L. major*. We demonstrate that changes in both parasite surface molecules and complement receptors available for parasite recognition and response result in alterations in phagocytosis, monokine production and T-cell cytokine production, thus potentially influencing the course of *L. major* infection *in vivo*.

METHODS

Mice

Adult female C57/BL6 mice (6 - 10 week old) were procured from the National Cancer Institute. Mice were kept in colonies at Colorado State University, under supervision of the CSU Laboratory Animal Resources Department with authorization by the Animal Care and Use Committee.

Parasites

L. major parasites from the LV39 MRHO/Sv/59/P (wild type), A2 (WT) and gp63KO (Joshi et al., 1998; Joshi et al., 2002) strains were propagated on sheep blood agar in parasite growth medium [RPMI-1640 cell culture medium (Sigma-Aldrich, St. Louis, MO) with 5% heat-inactivated fetal bovine serum (FBS) (Hyclone, Logan, UT), 10mM Hepes (Sigma-Aldrich), 100U/mL penicillin (Gibco, Carlsbad, CA), 100μg/mL streptomycin (Gibco), 2mM L-glutamine (Gibco), 1mM sodium pyruvate (Gibco), 0.2mM L-asparagine (Calbiochem, San Diego, CA), 0.6mM L-arginine (Calbiochem), and 2% sterile-filtered normal human urine]. *L. major* parasites from the LPGKO strain (Späth et al., 2000) were grown on sheep blood agar in identical medium, with the addition of 15 μg/mL of hygromycin (Gibco). Parasites were passaged through mice (susceptible BALB/c mice were infected with parasites, lesions were allowed to develop, then parasites were isolated from the lesions) every 2 months to retain virulence and from flask to flask twice a week. At 5 - 6 days after flask inoculation, parasites were removed from the growth medium, centrifuged to remove dead parasites and debris, washed in DMEM wash [as described above] and counted on a Neubauer hematocytometer prior to re-suspending in DMEM wash + 1% non-heat inactivated normal mouse serum (NMS) at

$50x10^6$ parasites per mL. Parasites were incubated for a minimum of 15 minutes in DMEM + 1% NMS prior to adding to plated macrophages to ensure complement opsonization.

Gp63KO parasites were developed from the *L. major* strain NIH S (MHOM/SN/74/Seidman) clone A2 parasite as described in Joshi et al. (1998, 2002). LPGKO parasites were developed from the *L. major* strain LV39 (MRHO/Sv/59/P) Clone 5 parasite as described in Späth et al. (2003). Cultures of these parasites were routinely checked for lack of gp63 or LPG expression by Western blot analysis of parasite lysates.

Intraperitoneal Macrophage Collection

Mice were anesthetized with intraperitoneal (IP) Ketamine (75 mg/kg) and Xylazine (15 mg/kg) prior to IP inoculation with 3 mL of saturated potato starch solution (2% w:v in PBS). Mice were euthanized via CO_2 inhalation after 3 days. The abdominal cavities were flushed 2-3 times for 3-4 minutes with 10mL of ice-cold DMEM+10% FBS [DMEM, 10mM Hepes, 100U/mL penicillin, 100μg/mL streptomycin, 2mM L-glutamine, 1mM sodium pyruvate, 0.2mM L-asparagine, 0.6mM L-arginine and 10% heat-inactivated fetal bovine serum]. The DMEM+10% FBS was aspirated and pooled on ice. The solution was spun at 300g for 10 minutes & the supernatant removed and discarded. The pellet was resuspended in DMEM+10% FBS. The spin & resuspension were repeated twice for a total of 3 times. The pellet was resuspended in a small amount of DMEM+10% FBS and viable cells counted using the trypan blue exclusion method. Cells gained by this method have been shown to be 95%+ macrophages (Titus et al., 1984). Volume was adjusted to give a concentration of 1 x 10^6 macrophages per mL and cells were aliquoted into 24-well plates (1 mL/well). Blocking antibodies were added at

this time. Blocking antibodies were kept on cells for 2 hours prior to adding parasites. *L. major* parasites were added to wells at $5x10^6$ parasites/well, in a 100 μL volume, for a total of 5 parasites per macrophage.

Bone marrow macrophage collection

Bone marrow derived macrophages were utilized to provide a un-stimulated macrophage population for assessment of phagocytosis based solely on phagocytic stimulus from *L. major*. Bone marrow macrophages were derived as described elsewhere (Kelso et al., 1982; Titus et al., 1984). Briefly, tibial bone marrow cells were cultured for 8-10 days in medium supplemented with either L-929 (mouse L cell)-conditioned medium as a source of macrophage colony stimulating factor (M-CSF) or 10-40 μg/mL recombinant murine M-CSF (BD-Biosciences). This approach is known to generate greater than 99% macrophages with no detectable lymphocytes and the sole contaminants being immature and mature cells of the granulocyte lineage (Kelso et al., 1982).

Determining phagocytosis and level of infection of bone marrow macrophages with Leishmania

To ensure that *Leishmania* successfully infected bone marrow macrophages from experiment to experiment, bone marrow macrophages derived from C57/BL6 mice were cultured as described (Titus et al., 1984). Briefly, bone marrow macrophages were allowed to adhere for 2 to 4 hrs to sterile coverslips in the wells of 24-well plates (3524, Costar), then blocking antibodies (as described in this section) were added. The bone marrow macrophages were then exposed to different infection ratios of *L. major* (see text) for 24 hrs. At this point the coverslips were removed and stained with Giemsa stain

(Behin et al., 1979). The stained preparations were examined microscopically and the number of intracellular parasites per 100 BM MØs was determined.

$CD4^+$ T-cell Isolation

Mice were euthanized via CO_2 inhalation. Spleens were collected and placed immediately on ice in 5 mL ice-cold PBS. Spleens were macerated and passed through a sterile wire mesh screen. The mesh screen was washed with 5 mL of ice cold PBS and removed. The splenic cell suspension was pipeted with a Pasteur pipet multiple times to break up cell clumps, then brought up to a volume of 10mL per spleen with more ice cold PBS and spun at 300g for 10 minutes at 4^o C. The supernatant was removed and cells were resuspended in ice-cold PBS. This was repeated twice more, then cells were passed through a 30μm filter and suspended in PBS. Viable cells were counted using the trypan blue exclusion method, then centrifuged at 300g for 10 minutes. Supernatant was discarded and cells resuspended in AutoMACs running buffer (PBS at pH 7.2, 2mM EDTA, 0.5% bovine serum albumin) (Miltenyi Biotec, Auburn, CA) at 10^7 cells per 40μL. Utilizing a commercially available CD4+ T-cell isolation kit & protocols (Miltenyi Biotec), CD4+ T-cells were isolated in an AutoMACS machine (Miltenyi Biotec). Recovered CD4+ T-cells (96+% purity) were washed twice in DMEM+10% FBS, counted and resuspended in DMEM+10% FBS at 2.5 $x10^6$ cells/mL (Matheu and Cahalan, 2007). T-cells were then added to macrophages in 24 well plates for co-culture experiments.

Blocking Antibodies

Antibodies used for blocking were anti-mouse CD21/CD35 (CR1/CR2) antibody, anti-mouse CD21 (CR1) antibody, and anti-mouse CD11b/CD18 (CR3) antibody (Boeringer-Mannheim). Antibodies were added at concentrations of 20μg/mL, as used in previous experiments (Puentes et al., 1988; da Silva et al., 1989). Antibodies were diluted in DMEM wash. Antibodies were added to macrophages immediately after they were placed in the wells and then incubated in a 37° C incubator with 5% CO_2 for 2 hours before adding parasites.

Cytokine Assays

Macrophages were co-cultured with *L. major* promastigotes for 24 to 48 hours. Plates were then centrifuged at 300g for 10 minutes and the supernatant collected and stored at -20° C. Commercially available ELISA's were performed to determine levels of IL-6, IL-12, TNF-α, and IL-10 (BD Pharmingen, San Jose, CA) in supernatants. Samples were assayed for levels of NO utilizing the Griess reaction (Sigma-Aldrich, St. Louis, MO).

In parallel experiments, macrophages were co-cultured with *L. major* promastigotes for 24 to 48 hours then macrophage plates were not centrifuged, but were washed 3 times with 37°C DMEM wash to remove un-phagocytosed parasites. Then, 1 mL of 37°C DMEM+10%FBS with 2.5 x10^6 $CD4^+$ T cells was added to each well. Macrophages and CD4+ T-cells were co-cultured at 37° C in 5% CO_2 for 5-7 days, at which time the plates were centrifuged at 300g and supernatants were collected and stored at -20° C. Commercially available ELISA's were used to determine levels of IFN-γ, IL-12, IL-4, TNF-α, and IL-10 (BD Pharmingen, San Jose, CA) in supernatants.

Samples were assayed for levels of NO utilizing the Griess reaction (Sigma-Aldrich, St. Louis, MO).

Statistical analysis

The Students paired *t* test and analysis of variance (ANOVA) were used for statistical analyses of the data (Systat 8.0 & SPSS 16.0 (Systat Software, Inc., Chicago, IL & SPSS, Inc., Chicago, IL). Differences were considered significant when $p < 0.05$.

RESULTS

Results are representative of pooled results from multiple replicate experiments. Blocking of CR1 and CR1/CR2 produced statistically indistinguishable results in all experiments where this was done. Thus, CR1 blocking is shown and CR1/CR2 blocking is not shown, in the interest of simplifying the affected graphs. Trends were similar for all of the experiments performed.

Phagocytosis of L. major by bone marrow macrophages co-cultured with complement receptor-blocking antibodies

L. major promastigotes from the A2, LV39, gp63KO and LPGKO strains were phagocytosed by macrophages in varying degrees following co-culture with macrophages (Figure 2.1 and 2.2). A2 and LV39 *L. major* strains had essentially similar degrees of phagocytosis (no significant differences seen between phagocytosis rates) and thus were pooled together as WT. Unblocked macrophages (with no blocking antibodies added) had significantly decreased ($p < 0.05$) levels of *L. major* phagocytosis seen when co-cultured with LPGKO or gp63KO parasites (Figure 2.2), which seemed to be ineffective compared to WT parasites at entering the macrophages (Figure 2.1 and 2.2). Gp63KO parasites did have an increased degree ($p < 0.05$) of phagocytosis compared to LPGKO parasites, albeit not at the level of WT parasites.

Macrophages that were pre-blocked with anti-CR antibodies (CR1, CR3, or CR1 and CR3 combined) had significantly decreased ($p < 0.05$) levels of phagocytosis of the WT strains of *L. major* compared to unblocked macrophages. Macrophages blocked with CR1 antibodies alone had higher levels of phagocytosis of WT *L. major* than those blocked with CR3 antibodies alone or CR1 and CR3 antibodies combined. There was no

significant difference seen between the phagocytosis rates of WT *L. major* for CR3 antibodies alone or CR1 and CR3 antibodies combined.

Macrophages blocked with CR1 antibodies alone or CR1 and CR3 antibodies combined had lower levels of phagocytosis of gp63KO *L. major* than those blocked with CR3 antibodies alone. In contrast, unblocked macrophages and CR3-blocked macrophages had similar rates of phagocytosis of gp63KO parasites. The rate of phagocytosis by CR3-blocked macrophages was also similar for WT and gp63KO parasites.

Macrophages that were pre-blocked with anti-CR3 antibodies alone or CR1 and CR3 antibodies combined had significantly decreased ($p < 0.05$) levels of LPGKO parasite phagocytosis compared to unblocked or CR1-blocked macrophages. Unblocked macrophages and macrophages blocked with anti-CR1 antibodies alone had similar levels of phagocytosis of LPGKO *L. major*.

Briefly, LPGKO parasites had the lowest phagocytosis rates overall, with gp63KO parasites falling between LPGKO and WT parasites. Blocking of CR1 and CR3 combined generally produced the greatest reduction in parasite phagocytosis for all parasite strains. Blocking of CR1 produced the least impact on phagocytosis rates for WT and LPGKO parasites, but for gp63KO parasites, CR1 blocking had a greater impact than CR3 blocking. Blocking of CR3 was equivalent to blocking both CR1 and CR3 for WT parasites and produced phagocytosis rates for LPGKO parasites that were between those of CR1-blocked and CR1 and CR3-blocked macrophages. There was no significant effect from blocking CR3 on gp63KO phagocytosis compared to unblocked macrophages. CR3-blocked WT parasites had equal levels of phagocytosis to CR3

blocked gp63KO parasites, unlike all other CR blocking in either gp63KO or LPGKO strains.

These differences between the *L. major* strains persisted in groups with varying times of parasite co-culture (6-24 hours) and varying ratios of numbers of parasites added per macrophage (2:1 to 100:1).

Cytokine production by L. major-infected bone marrow macrophages co-cultured with complement receptor-blocking antibodies

ELISA assays were performed in triplicate for each experiment and multiple experiments were performed and pooled. Nitric oxide (NO), IL-4, and IFN-γ assays were performed, but showed no significant effects or differences between any of the treatment groups (data not shown).

Fold increase in cytokine levels of IL-6, IL-10, IL-12, and TNF-α are shown for each combination of parasite strain and complement receptor blocking in Tables 2.1 and 2.2, with statistically significant differences between results noted. Figures 2.3A-2.3D provide a graphical view of the relative increases in cytokine levels produced by macrophages infected with the three separate parasite strains combined with the different complement receptor blockers. Figures 2.4A-2.4D provide a graphical view of the relative increases in cytokine levels produced by naïve T-cells co-cultured with macrophages infected with the three separate parasite strains combined with the different complement receptor blockers.

Macrophage-derived IL-6 had slightly elevated levels seen in CR1 and CR1+CR3 blocked groups for all strains compared to unblocked groups, particularly at 48 hours, with only CR1-blocked groups having elevated levels over unblocked controls at 72

hours (Table 2.1A and Figure 2.3A). Overall, IL-6 levels tended to increase slightly or remain steady from the 48 to 72 hour timepoints, although LPGKO and gp63KO parasites had lower levels compared to WT at 48 hours for unblocked, CR1, CR3 (gp63KO only) and CR1+CR3 blocked macrophages and for only the CR1 (gp63KO only) and CR1+CR3 blocked macrophages at 72 hours. In co-culture experiments, when CR3 alone or CR1+CR3 were blocked, IL-6 levels from WT-infected and LPGKO-infected groups increased at 24 hour and 48 hour timepoints (Table 2.2A and Figure 2.4A). At the 24 hour timepoint, both LPGKO and gp63KO infections tended to produce lower IL-6 levels than those seen in WT infections, regardless of the CR blocked, whereas at 48 hours, only gp63KO *L. major* combined with CR1-blocked macrophages had notably reduced IL-6 levels compared to WT. IL-6 levels from co-culture experiments were only slightly elevated overall at 24 hours, but were notably increased over those seen in the macrophage experiments at the 48 hour timepoint (Figure 2.3A and Figure 2.4A).

At 48 hours, macrophage-derived IL-10 levels were increased with CR1, CR3, and CR1+CR3-blocking in WT parasites and CR3 and CR1+CR3 blocking in LPGKO and gp63KO parasites over that seen with unblocked macrophages (Table 2.1A and Figure 2.3B). However, at 72 hours, macrophage-derived IL-10 levels were dramatically increased from the unblocked macrophages infected with all three *L. major* strains, while CR-blocking of any sort significantly reduced the IL-10 levels produced (Table 2.1A and Figure 2.3B). IL-10 levels were only slightly altered by CR-blocking or differences in *L. major* strain in the co-cultured experiments (Table 2.2A and Figure 2.4B).

Macrophage-derived IL-12 levels were highest in unblocked macrophages, increasing from the 48 hour to 72 hour timepoint (Table 2.1B and Figure 2.3C). CR blocking (CR1, CR3, or CR1+CR3) resulted in decreased IL-12 production compared to unblocked macrophages (Table 2.1B and Figure 2.3C). Generally, macrophages infected with gp63KO parasites produced the lowest IL-12 levels and those infected with LPGKO parasites had the highest (Table 2.1B and Figure 2.3C). In the 24 hour co-culture experiments, Il-12 levels were highest in unblocked macrophages infected with WT or LPGKO parasites, with CR blocking producing dramatically decreased IL-12 levels compared to unblocked groups in both LPGKO and WT-infected groups (Table 2.2B and Figure 2.4C). At 48 hours of co-culture, CR blocking produced notable decreases in IL-12 levels in both LPGKO and gp63KO groups, although not in WT-infected groups (Table 2.2B and Figure 2.4C).

At 48 hours, the macrophage-derived TNF-α levels seen in CR1 and CR3-blocked groups significantly increased over those seen in unblocked macrophages for all three parasite strains (WT, LPGKO, and gp63KO) (Table 2.1B and Figure 2.3D). TNF-α levels were higher in CR1-blocked macrophages than CR3-blocked or CR1+CR3-blocked macrophages (Table 2.1B and Figure 2.3D). LPGKO parasites had the lowest levels of TNF-α production at 48 hours, but still followed the same pattern of response overall (Table 2.1B and Figure 2.3D). This effect was not apparent at the later 72 hour timepoint (Table 2.1B and Figure 2.3D). The lack of a TNF-α response persisted in the co-culture experiments, with an overall very limited TNF-α response (Table 2.2B and Figure 2.4D).

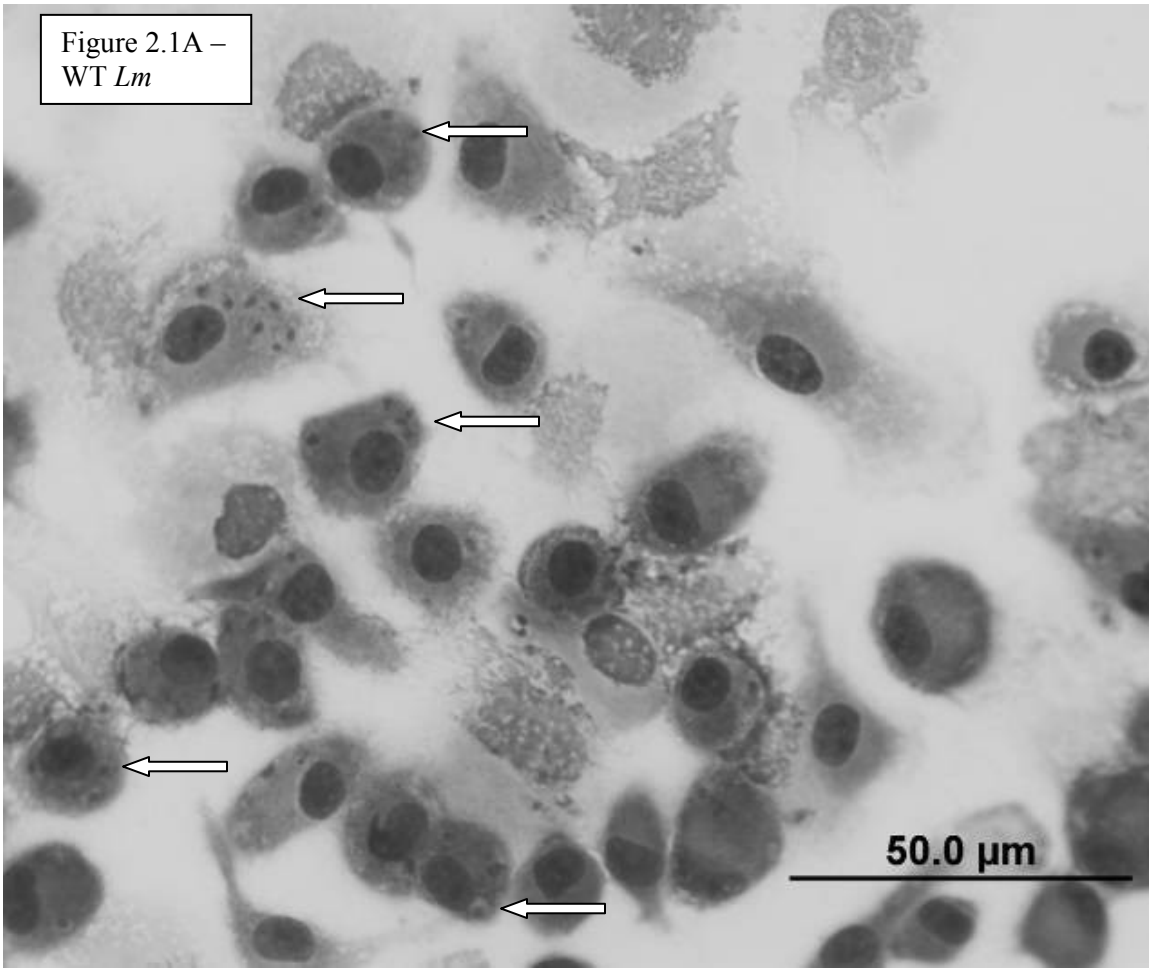

Figure 2.1A – Phagocytosed *L. major* of WT strain within macrophages (without complement receptors blocked). White arrows point to amastigote-stage parasites within macrophages. Note the decreased numbers of intracellular *L. major* seen in the LPGKO (B) and gp63KO (C) images. Giemsa stained slides, 200x.

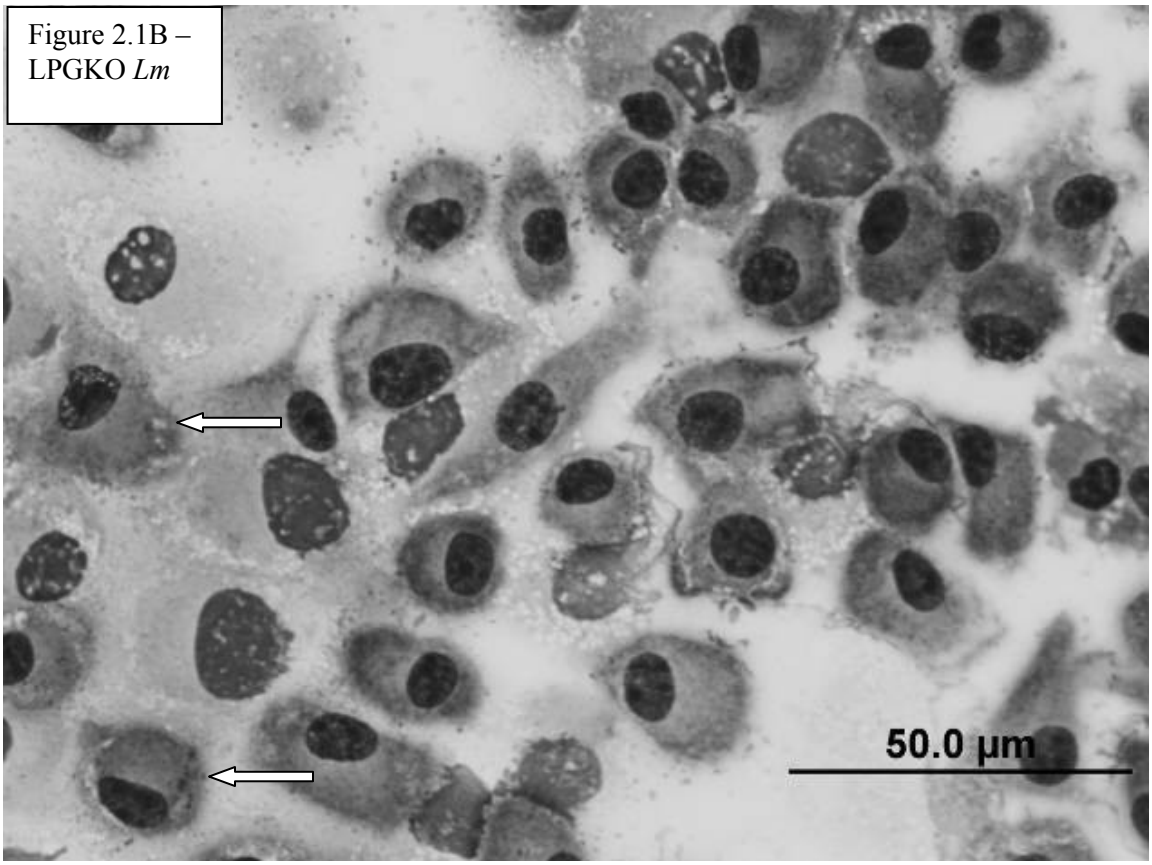

Figure 2.1B – Phagocytosed *L. major* of LPGKO strain within macrophages (without complement receptors blocked). White arrows point to amastigote-stage parasites within macrophages. Note the decreased numbers of intracellular *L. major* seen in the LPGKO (B) and gp63KO (C) images. Giemsa stained slides, 200x.

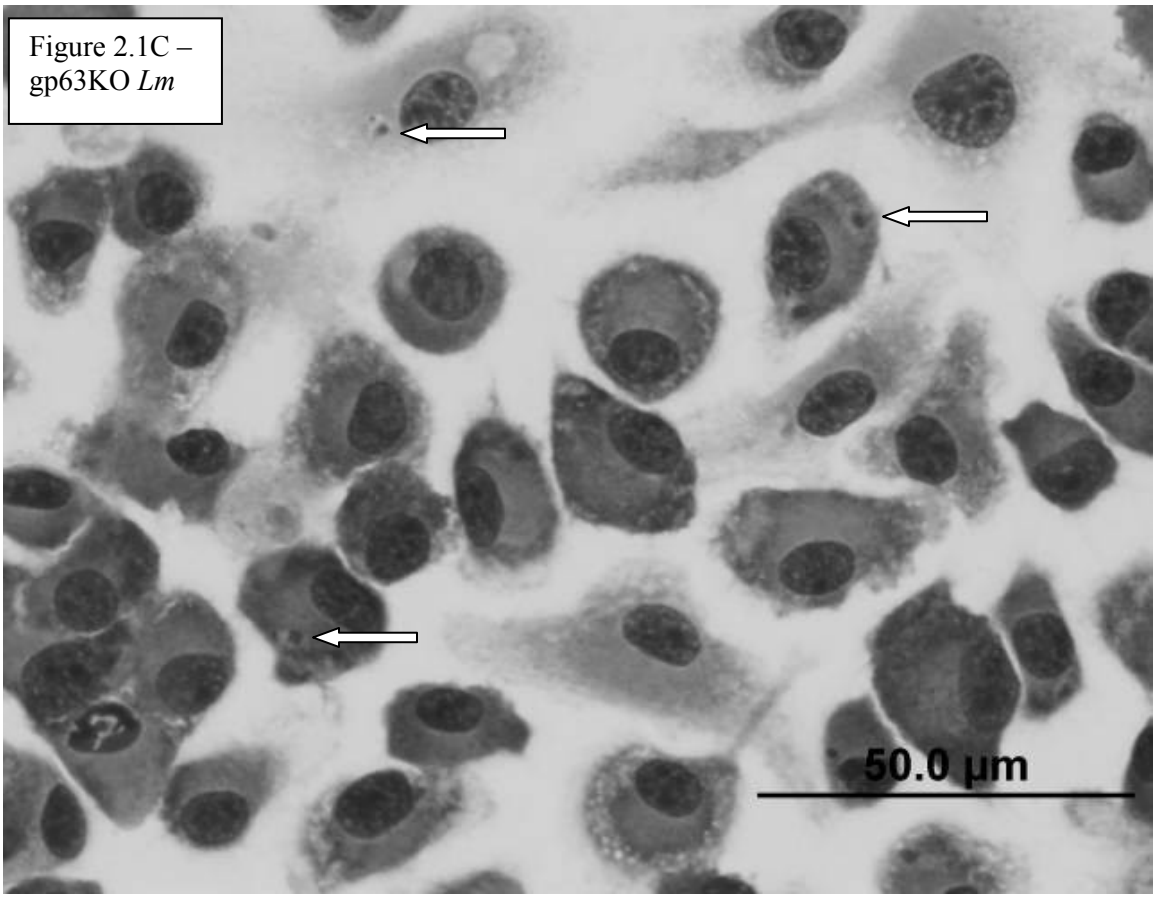

Figure 2.1C – Phagocytosed *L. major* of gp63KO strain within macrophages (without complement receptors blocked). White arrows point to amastigote-stage parasites within macrophages. Note the decreased numbers of intracellular *L. major* seen in the LPGKO (B) and gp63KO (C) images. Giemsa stained slides, 200x.

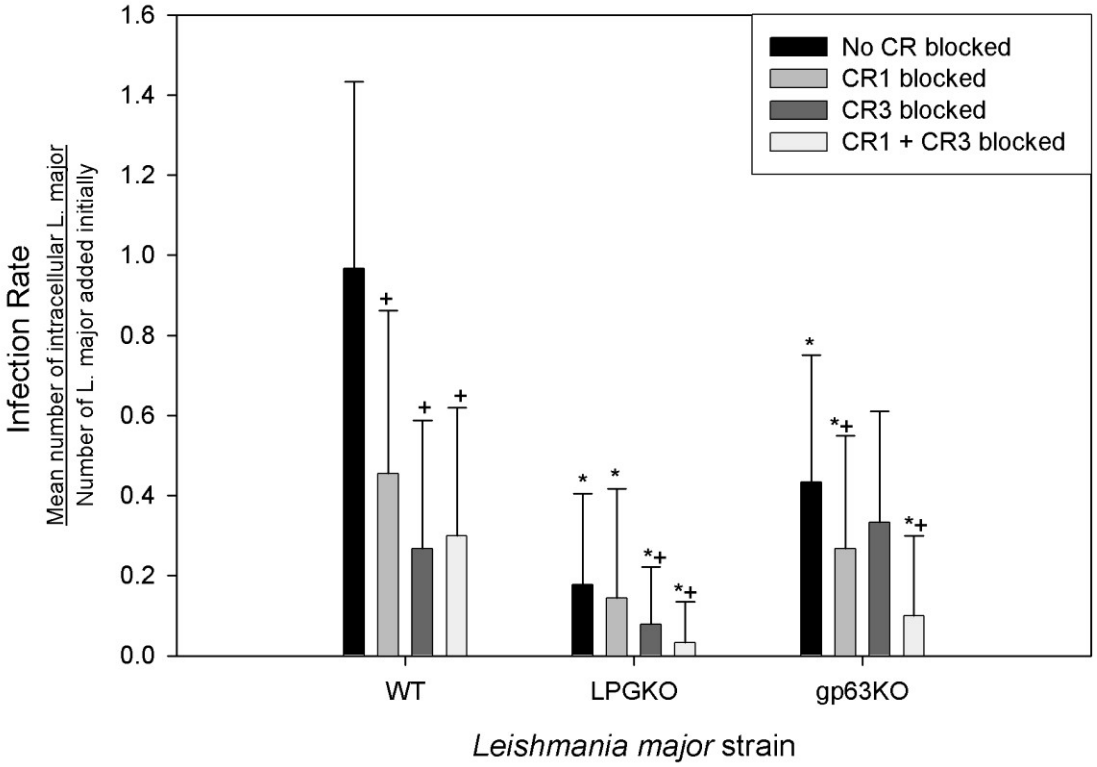

Figure 2.2 – Infection rate in parasites per macrophage of macrophages by *L. major* of wild type (WT), LPGKO or gp63KO strains, with or without blocking of complement receptors (CR1, CR3, CR1+CR3). Error bars show standard deviation. Complement receptor blocked groups marked with $^{+}$ are significantly different ($p < 0.05$) from control groups using the same strain of *L. major*, with no blocking of complement receptors. LPGKO-infected or gp63KO-infected unblocked or complement receptor blocked groups marked with * are significantly different ($p < 0.05$) from the similarly blocked control groups using the WT strain of *L. major*.

Table 2.1A – Levels of monokines (IL-6, IL-10) produced by macrophages infected by *Leishmania major* of wild type (WT), LPGKO or gp63KO strains, with or without blocking of complement receptors (CR1, CR3, CR1+CR3). Monokine production is expressed in fold differences compared to those produced by similarly blocked or unblocked macrophages that were not exposed to *L. major*. Macrophages were first blocked with anti-CR antibodies for 2 hours, then incubated for either 48 hours or 72 hours with *L. major*.

Table 2.1A: Macrophage produced cytokines (IL-6 and IL-10)

			48 Hours Post-infection		72 Hours Post-infection	
Cytokine	**Parasite Strain**	**CR Blocking**	**Fold Increase**	**SD**	**Fold Increase**	**SD**
IL-6	WT	None	**2.3**	0.5	**4.0**	2.4
		CR1	**4.1** [3,5]	0.6	**6.8** [3]	1.3
		CR3	**1.9** [4]	0.8	**6.9**	3.1
		CR1+CR3	**4.2** [3,4,5]	0.6	**4.4** [3,4,5]	0.01
	LPGKO	None	**1.2** [1]	0.1	**4.1**	3.3
		CR1	**1.6** [1,3,5]	0.1	**6.9** [3]	4.8
		CR3	**1.5** [3,4]	0.2	**4.3**	0.2
		CR1+CR3	**3.1** [1,3,4,5]	1.1	**2.9** [1,3]	0.01
	Gp63KO	None	**0.9** [1,2]	0.2	**2.5**	1.3
		CR1	**1.0** [1,2,3]	0.2	**4.9** [1,3]	2.1
		CR3	**1.0** [1]	0.9	**4.3**	0.7
		CR1+CR3	**2.3** [1,3,4,5]	0.1	**2.8** [1,5]	0.2
IL-10	WT	None	**3.2**	2.0	**102.8**	116.8
		CR1	**4.2** [3,5]	2.3	**14.2**	15.2
		CR3	**10.9** [3,4]	4.0	**3.9**	2.7
		CR1+CR3	**11.7** [3,4,5]	4.2	**0.8** [5]	0.1
	LPGKO	None	**4.7**	3.9	**53.1**	60.2
		CR1	**4.1** [5]	3.4	**8.2**	9.1
		CR3	**8.1** [1,3,4]	5.5	**2.7** [1]	2.4
		CR1+CR3	**9.1** [1,3,4,5]	5.3	**0.4** [3]	0.01
	Gp63KO	None	**1.7** [1]	1.1	**59.5**	67.7
		CR1	**3.6** [5]	3.0	**9.7**	10.5
		CR3	**9.0** [1,3,4]	2.7	**3.7** [2]	2.9
		CR1+CR3	**12.4** [2,3,4]	5.6	**0.9** [5]	0.1

1 = significantly different ($p < 0.05$) from WT strain with same blocking antibodies
2 = significantly different ($p < 0.05$) from LPGKO strain with same blocking antibodies
3 = significantly different ($p < 0.05$) from same strain with no blocking antibodies
4 = significantly different ($p < 0.05$) from same strain blocked with CR1 antibodies
5 = significantly different ($p < 0.05$) from same strain blocked with CR3 antibodies

Fold Increase = Increase in cytokine levels over that produced by similarly treated macrophages that were not exposed to *L. major*
SD = Standard Deviation
CR = Complement Receptor

Table 2.1B – Levels of monokines (IL-12, TNF-α) produced by macrophages infected by *Leishmania major* of wild type (WT), LPGKO or gp63KO strains, with or without blocking of complement receptors (CR1, CR3, CR1+CR3). Monokine production is expressed in fold differences compared to those produced by similarly blocked or unblocked macrophages that were not exposed to *L. major*. Macrophages were first blocked with anti-CR antibodies for 2 hours, then incubated for either 48 hours or 72 hours with *L. major*.

Table 2.1B: Macrophage produced cytokines (IL-12 and TNF-α)

			48 Hours Post-infection		72 Hours Post-infection	
Cytokine	**Parasite Strain**	**CR Blocking**	**Fold Increase**	**SD**	**Fold Increase**	**SD**
IL-12	WT	None	**112.0**	64.2	**494.9**	68.8
		CR1	**59.5**	12.5	**61.2** [3]	16.4
		CR3	**38.6** [4]	17.3	**56.7**[3]	11.8
		CR1+CR3	**4.6** [3,4,5]	2.8	**---**	--
	LPGKO	None	**217.1** [1]	114.8	**664.0** [1]	38.5
		CR1	**37.9** [1,3]	11.0	**67.7**[3]	1. 5
		CR3	**63.1** [3]	18.0	**89.7** [1,3]	26.5
		CR1+CR3	**12.1** [1,3,4,5]	3.4	**---**	--
	Gp63KO	None	**40.0**[1,2]	0.02	**514.7** [2]	40.9
		CR1	**6.8** [1,2,3]	3.7	**40.3** [1,2,3]	13.2
		CR3	**11.3** [2,3]	10.7	**50.4** [3]	6.0
		CR1+CR3	**2.0** [2,3,4,5]	0.01	**---**	--
TNF-α	WT	None	**3.6**	0.04	**3.3**	0.1
		CR1	**37.6** [3]	8.7	**3.8** [5]	0.02
		CR3	**17.0** [4]	15.2	**3.4** [4]	0.0
		CR1+CR3	**15.6** [3,4]	7.5	**2.7** [3,4,5]	0.1
	LPGKO	None	**2.0** [1]	0.1	**1.4** [1]	0.04
		CR1	**22.9** [1,3]	19.2	**1.4** [1]	0.04
		CR3	**12.2** [1,3,4]	10.8	**1.3** [1]	0.01
		CR1+CR3	**8.0** [1,3]	4.0	**1.2** [1,5]	0.0
	Gp63KO	None	**2.2** [1]	0.1	**1.7** [1]	0.1
		CR1	**26.2** [3]	23.9	**1.5**[1]	0.01
		CR3	**15.8** [3,4]	15.4	**1.9** [1,4]	0.1
		CR1+CR3	**14.5** [3,4]	15.2	**1.4** [1,2]	0.02

1 = significantly different ($p < 0.05$) from WT strain with same blocking antibodies
2 = significantly different ($p < 0.05$) from LPGKO strain with same blocking antibodies
3 = significantly different ($p < 0.05$) from same strain with no blocking antibodies
4 = significantly different ($p < 0.05$) from same strain blocked with CR1 antibodies
5 = significantly different ($p < 0.05$) from same strain blocked with CR3 antibodies

Fold Increase = Increase in cytokine levels over that produced by similarly treated macrophages that were not exposed to *L. major*
SD = Standard Deviation
CR = Complement Receptor

Table 2.2A – Levels of cytokines (IL-6, IL-10) produced by cocultures of naïve, undifferentiated T cells with macrophages infected by *Leishmania major* of wild type (WT), LPGKO or gp63KO strains, with or without blocking of complement receptors (CR1, CR3, CR1+CR3). Cytokine production is expressed in fold differences compared to those produced by similarly blocked or unblocked co-cultures that were not exposed to *L. major*. Macrophages and T-cells were co-cultured for either 24 hours or 48 hours following macrophage blocking by anti-CR antibodies and 5 days of infection by *L. major*.

Table 2.2A: T-cell/Macrophage co-culture-produced cytokines (IL-6 and IL-10)

			24 Hours Co-culture		48 Hours Co-culture	
Cytokine	**Parasite Strain**	**CR Blocking**	**Fold Increase**	**SD**	**Fold Increase**	**SD**
IL-6	WT	None	**1.1**	0.3	**3.2**	0.2
		CR1	**4.3** [5]	0.1	**5.4**	3.8
		CR3	**10.1** [3,4]	0.6	**18.9**	15.1
		CR1+CR3	**7.6** [3,4]	0.2	**6.0** [3]	1.2
	LPGKO	None	**0.5** [1]	0.3	**0.7** [1]	0.7
		CR1	**2.0** [1]	0.1	**1.3** [5]	0.3
		CR3	**1.5** [1]	0.2	**4.7** [4]	1.9
		CR1+CR3	**5.3** [1,3,4,5]	0.1	**9.2** [3,4]	4.5
	Gp63KO	None	**1.7**	0.1	**3.7**	1.5
		CR1	**1.1** [1,2]	0.1	**0.8** [1,3]	0.0
		CR3	**1.8** [1]	0.2	**6.2**	3.1
		CR1+CR3	**1.6** [1,2]	0.03	**3.7** [4,5]	2.1
IL-10	WT	None	**2.4**	0.5	**1.6**	0.3
		CR1	**1.4**	0.9	**1.1**	1.0
		CR3	**1.3** [3]	0.3	**1.1**	0.6
		CR1+CR3	**1.1** [3]	0.4	**1.3**	0.3
	LPGKO	None	**2.0**	1.1	**0.7**	0.6
		CR1	**0.9** [1,3]	0.7	**1.1**	1.0
		CR3	**2.0**	0.9	**1.1** [3]	0.5
		CR1+CR3	**1.6**	0.5	**0.9**	0.5
	Gp63KO	None	**1.9**	0.1	**1.3** [2]	0.7
		CR1	**1.5** [2]	0.7	**1.2**	1.8
		CR3	**1.1** [2,3]	0.4	**1.5**	1.8
		CR1+CR3	**1.6** [5]	0.4	**1.2**	1.1

1 = significantly different ($p < 0.05$) from WT strain with same blocking antibodies
2 = significantly different ($p < 0.05$) from LPGKO strain with same blocking antibodies
3 = significantly different ($p < 0.05$) from same strain with no blocking antibodies
4 = significantly different ($p < 0.05$) from same strain blocked with CR1 antibodies
5 = significantly different ($p < 0.05$) from same strain blocked with CR3 antibodies

Fold Increase = Increase in cytokine levels over that produced by similarly treated macrophages that were not exposed to *L. major*
SD = Standard Deviation
CR = Complement Receptor

Table 2.2B – Levels of cytokines (IL-12, TNF-α) produced by cocultures of naïve, undifferentiated T cells with macrophages infected by *Leishmania major* of wild type (WT), LPGKO or gp63KO strains, with or without blocking of complement receptors (CR1, CR3, CR1+CR3). Cytokine production is expressed in fold differences compared to those produced by similarly blocked or unblocked co-cultures that were not exposed to *L. major*. Macrophages and T-cells were co-cultured for either 24 hours or 48 hours following macrophage blocking by anti-CR antibodies and 5 days of infection by *L. major*.

Table 2.2B: T-cell/Macrophage co-culture-produced cytokines (IL-12 and TNF-α)

			24 Hours Co-culture		48 Hours Co-culture	
Cytokine	**Parasite Strain**	**CR Blocking**	**Fold Increase**	**SD**	**Fold Increase**	**SD**
IL-12	WT	None	**40.2**	13.2	**5.0**	5.7
		CR1	**2.2** [3,5]	0.3	**7.0** [5]	2.8
		CR3	**5.4** [3,4]	0.8	**1.6** [4]	0.4
		CR1+CR3	**2.5** [3,5]	0.7	**1.7** [4]	0.3
	LPGKO	None	**24.2** [1]	9.5	**32.6**	44.3
		CR1	**1.7** [3]	0.3	**11.4**	14.7
		CR3	**3.8** [1,3]	1.4	**4.8** [1]	0.5
		CR1+CR3	**2.2** [1,3,4]	0.5	**1.8** [5]	0.4
	Gp63KO	None	**1.0** [1,2]	0.0	**35.0**	47.7
		CR1	**0.6** [1,2,5]	0.6	**28.6**	27.8
		CR3	**2.1** [1,2,3,4]	0.3	**1.6** [2]	0.4
		CR1+CR3	**1.2** [1,2,3,4,5]	0.2	**0.8** [1,2,5]	0.1
TNF-α	WT	None	**0.5**	0.5	**1.0**	0.04
		CR1	**0.9**	1.0	**0.8**	0.1
		CR3	**1.0** [3]	0.7	**1.5** [3]	0.0
		CR1+CR3	**0.6**	0.3	**1.3**	0.0
	LPGKO	None	**0.7**	0.6	**1.6**	0.1
		CR1	**0.7**	0.8	**0.7**	0.1
		CR3	**0.9**	0.5	**1.5**	0.03
		CR1+CR3	**1.4**	0.7	**0.5** [3]	0.2
	Gp63KO	None	**0.8** [1]	0.6	**1.7** [1]	0.1
		CR1	**1.9** [2,3,5]	0.3	**1.1** [2,3]	0.1
		CR3	**1.4** [1,2,3,4]	0.6	**1.8**	0.1
		CR1+CR3	**1.4** [1,3]	0.6	**1.3**	0.02

1 = significantly different ($p < 0.05$) from WT strain with same blocking antibodies
2 = significantly different ($p < 0.05$) from LPGKO strain with same blocking antibodies
3 = significantly different ($p < 0.05$) from same strain with no blocking antibodies
4 = significantly different ($p < 0.05$) from same strain blocked with CR1 antibodies
5 = significantly different ($p < 0.05$) from same strain blocked with CR3 antibodies

Fold Increase = Increase in cytokine levels over that produced by similarly treated macrophages that were not exposed to *L. major*
SD = Standard Deviation
CR = Complement Receptor

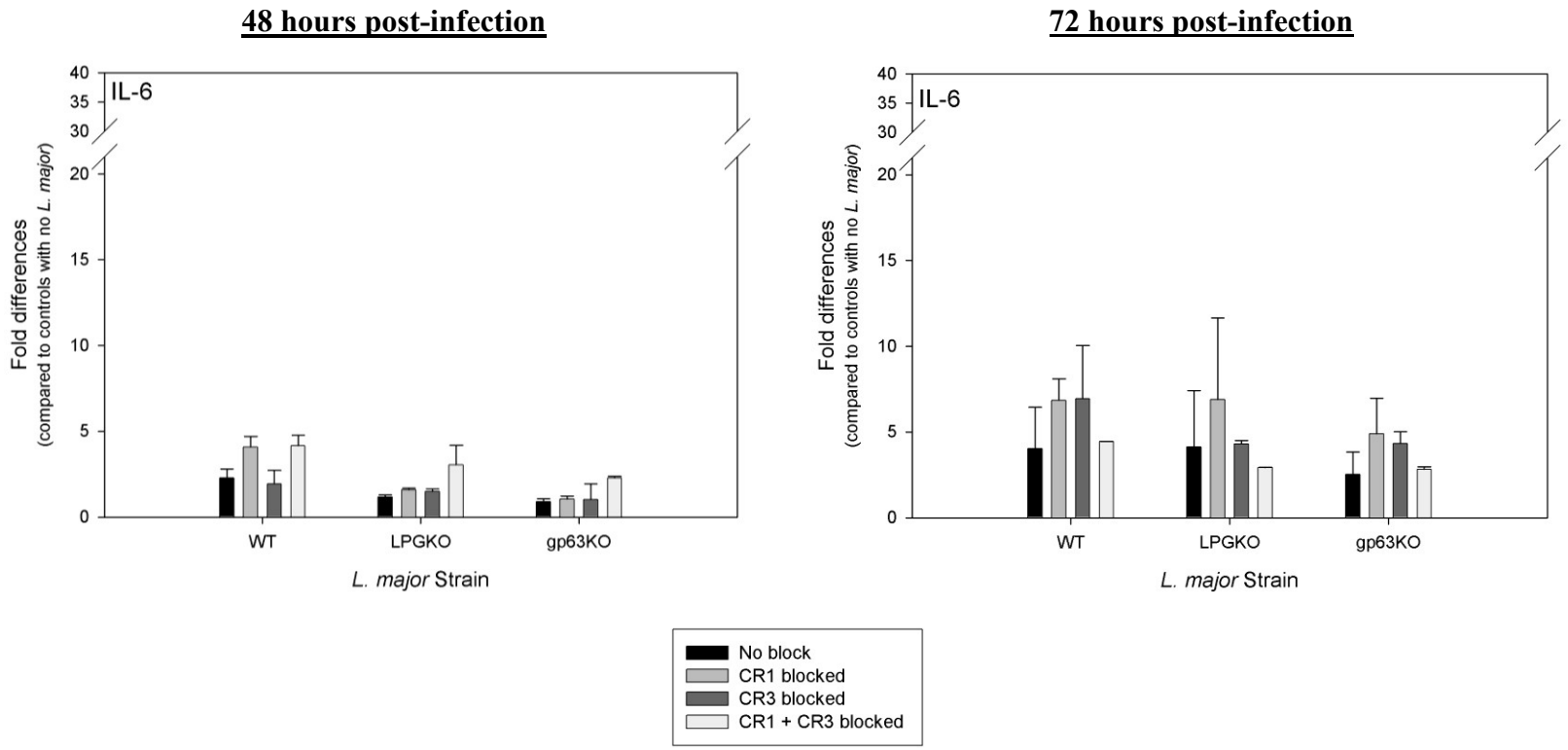

Figure 2.3A – Levels of IL-6 produced by macrophages infected by *Leishmania major* of wild type (WT), LPGKO or gp63KO strains, with or without blocking of complement receptors (CR1, CR3, CR1+CR3). Monokine production is expressed in fold differences compared to those produced by similarly blocked or unblocked macrophages that were not exposed to *L. major*. Macrophages were first blocked with anti-CR antibodies for 2 hours, then incubated for either 48 hours or 72 hours with *L. major*.

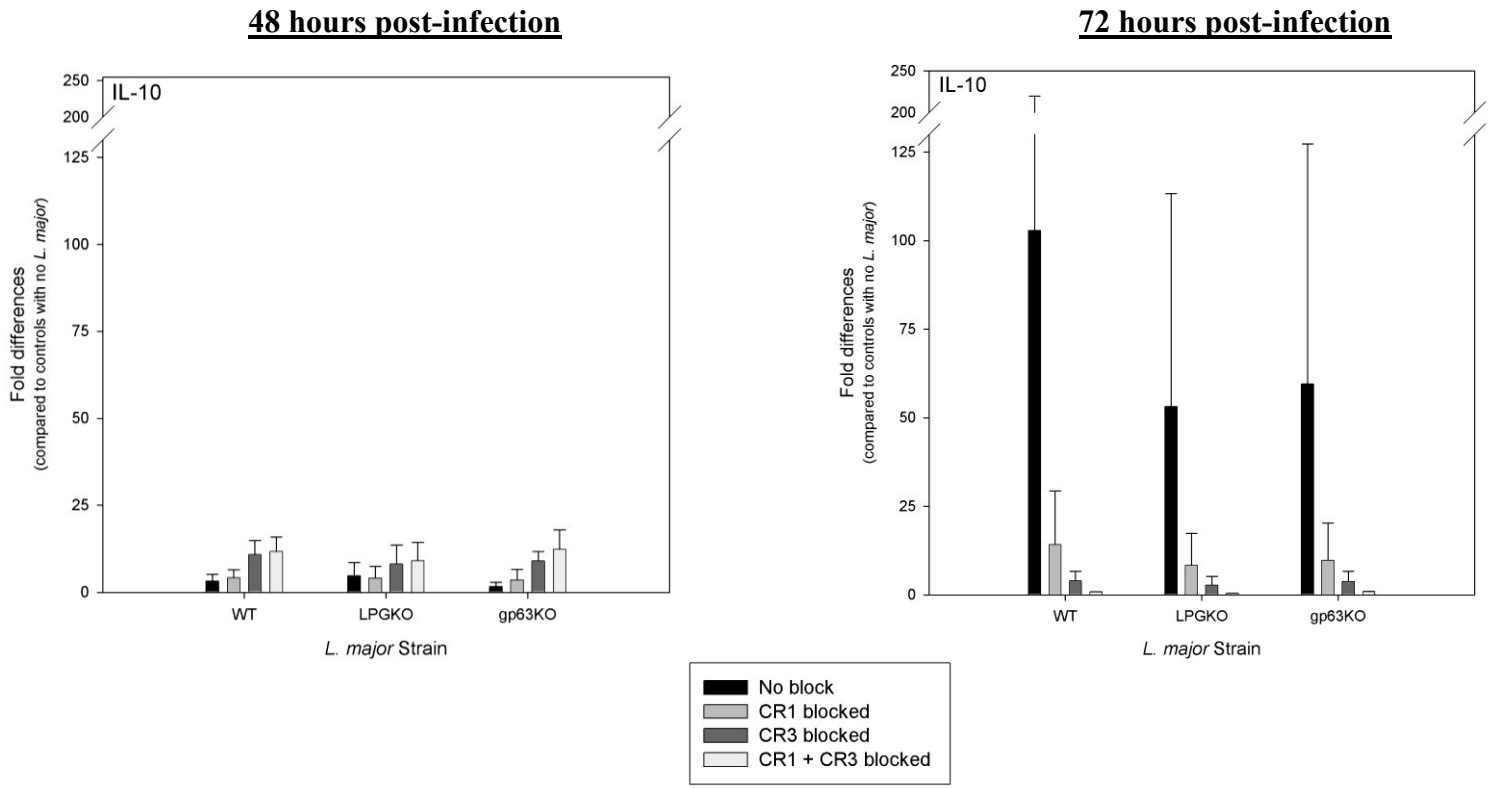

Figure 2.3B – Levels of IL-10 produced by macrophages infected by *Leishmania major* of wild type (WT), LPGKO or gp63KO strains, with or without blocking of complement receptors (CR1, CR3, CR1+CR3). Monokine production is expressed in fold differences compared to those produced by similarly blocked or unblocked macrophages that were not exposed to *L. major*. Macrophages were first blocked with anti-CR antibodies for 2 hours, then incubated for either 48 hours or 72 hours with *L. major*.

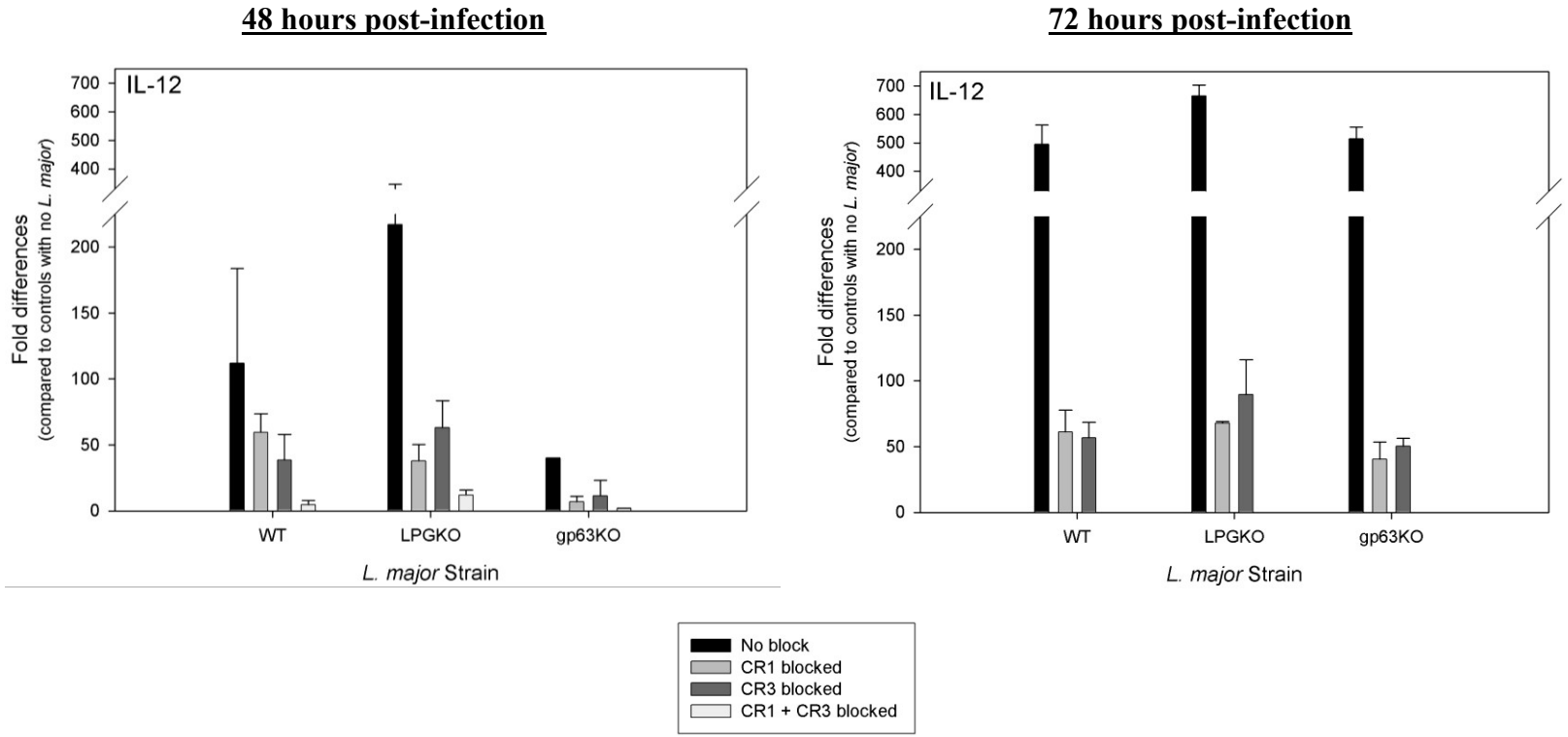

Figure 2.3C – Levels of IL-12 produced by macrophages infected by *Leishmania major* of wild type (WT), LPGKO or gp63KO strains, with or without blocking of complement receptors (CR1, CR3, CR1+CR3). Monokine production is expressed in fold differences compared to those produced by similarly blocked or unblocked macrophages that were not exposed to *L. major*. Macrophages were first blocked with anti-CR antibodies for 2 hours, then incubated for either 48 hours or 72 hours with *L. major*.

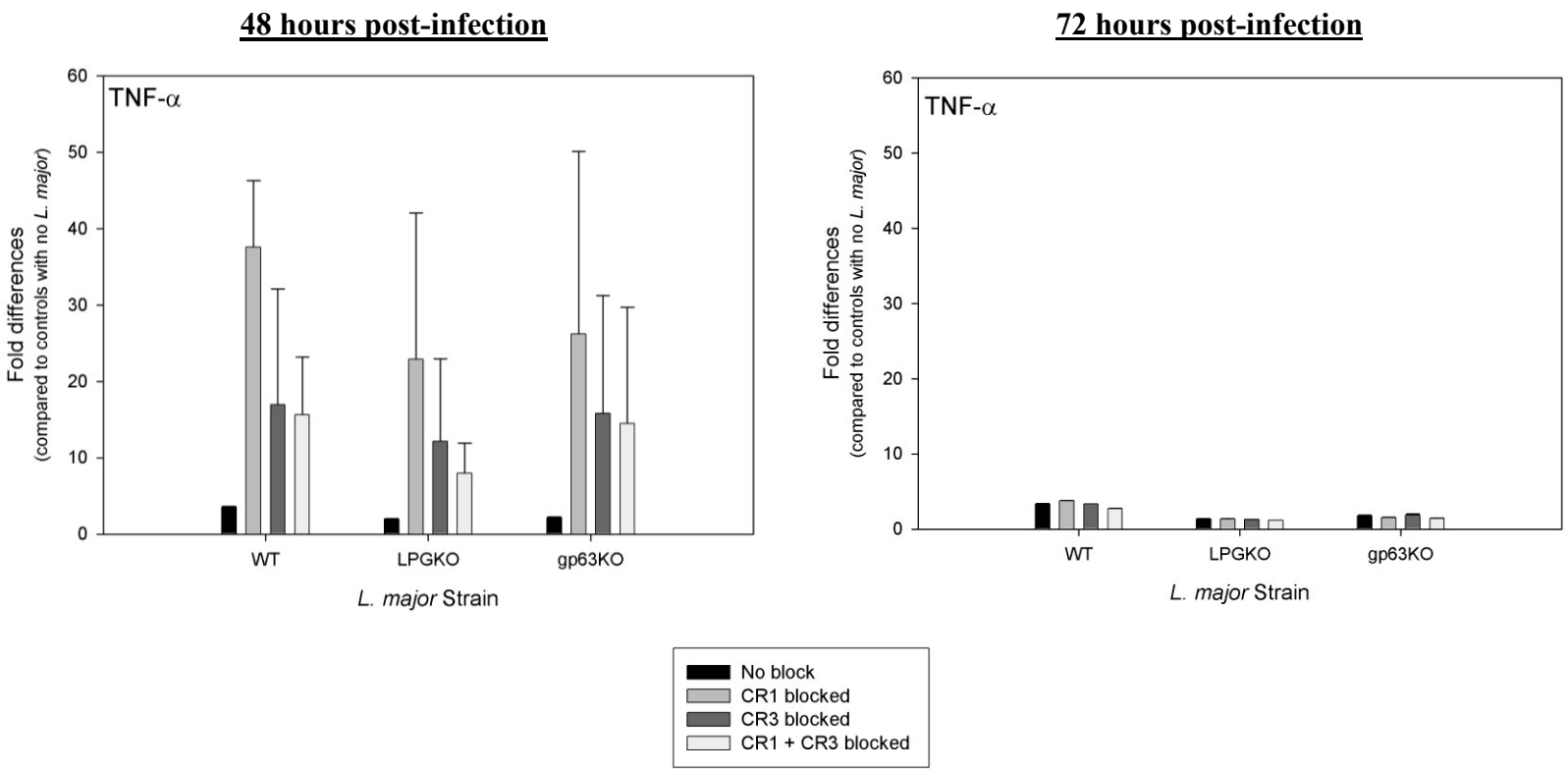

Figure 2.3D – Levels of TNF-α produced by macrophages infected by *Leishmania major* of wild type (WT), LPGKO or gp63KO strains, with or without blocking of complement receptors (CR1, CR3, CR1+CR3). Monokine production is expressed in fold differences compared to those produced by similarly blocked or unblocked macrophages that were not exposed to *L. major*. Macrophages were first blocked with anti-CR antibodies for 2 hours, then incubated for either 48 hours or 72 hours with *L. major*.

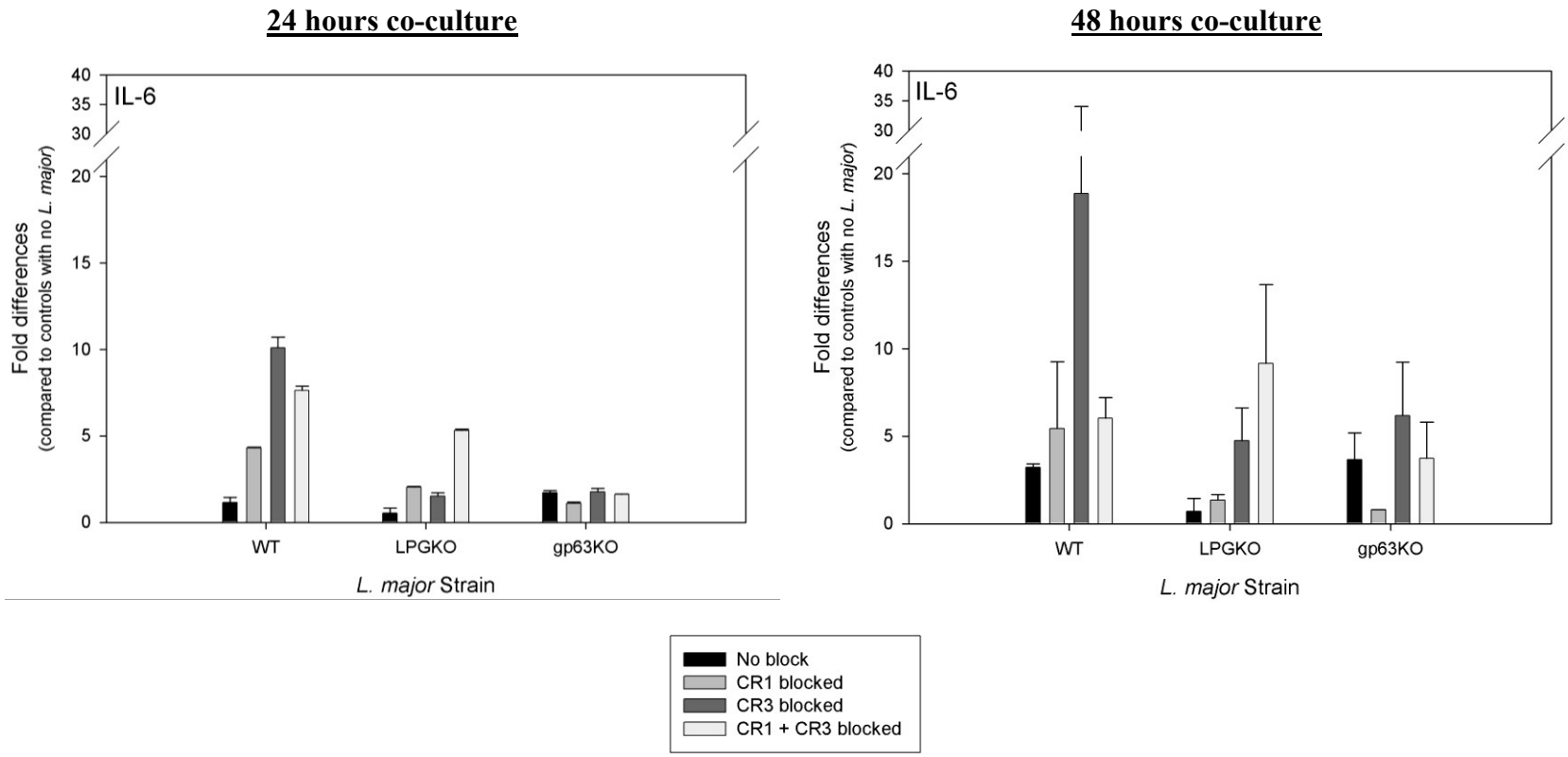

Figure 2.4A – Levels of IL-6 produced by cocultures of naïve, undifferentiated T cells with macrophages infected by *Leishmania major* of wild type (WT), LPGKO or gp63KO strains, with or without blocking of complement receptors (CR1, CR3, CR1+CR3). Cytokine production is expressed in fold differences compared to those produced by similarly blocked or unblocked co-cultures that were not exposed to *L. major*. Macrophages and T-cells were co-cultured for either 24 hours or 48 hours following macrophage blocking by anti-CR antibodies and 5 days of infection by *L. major*.

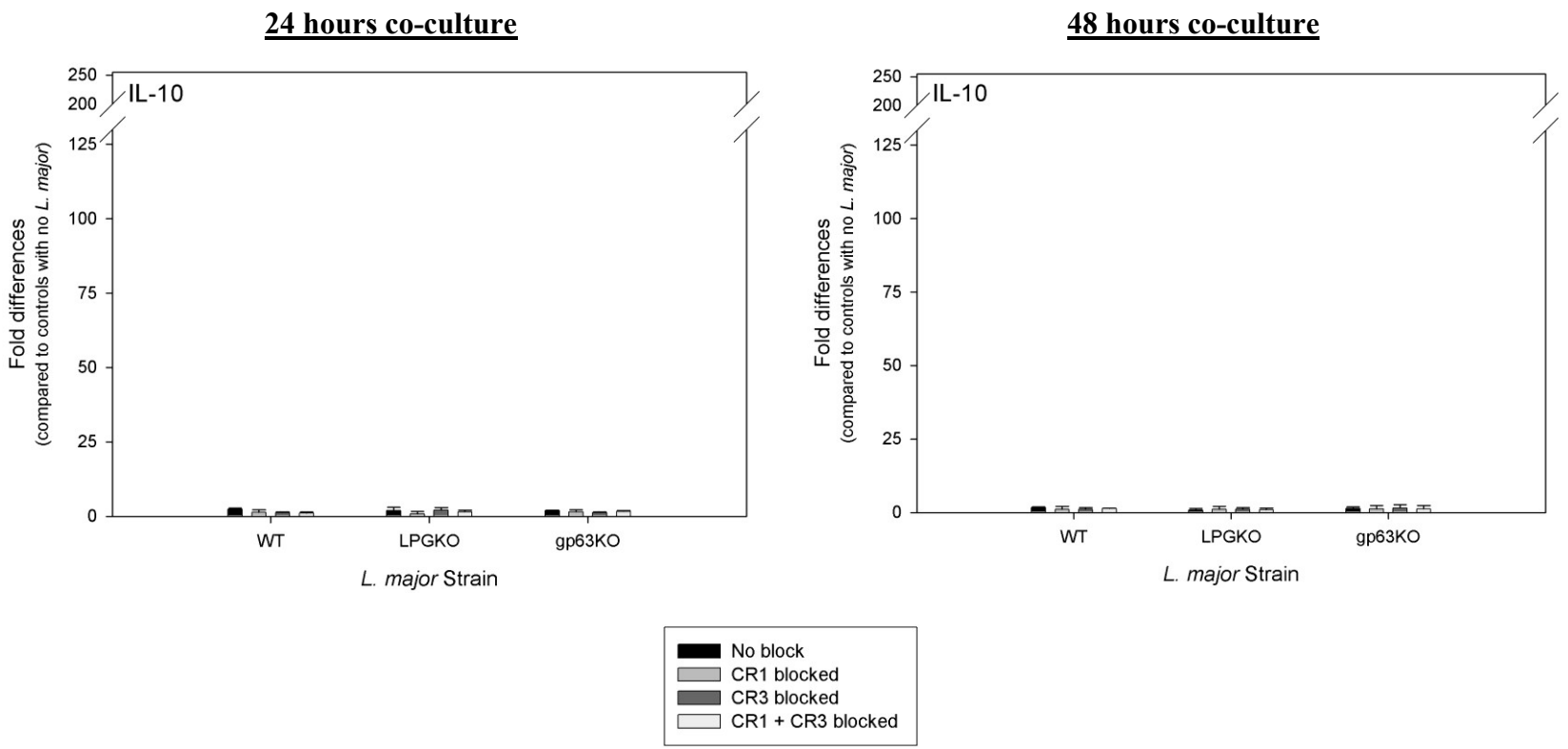

Figure 2.4B – Levels of IL-10 produced by cocultures of naïve, undifferentiated T cells with macrophages infected by *Leishmania major* of wild type (WT), LPGKO or gp63KO strains, with or without blocking of complement receptors (CR1, CR3, CR1+CR3). Cytokine production is expressed in fold differences compared to those produced by similarly blocked or unblocked co-cultures that were not exposed to *L. major*. Macrophages and T-cells were co-cultured for either 24 hours or 48 hours following macrophage blocking by anti-CR antibodies and 5 days of infection by *L. major*.

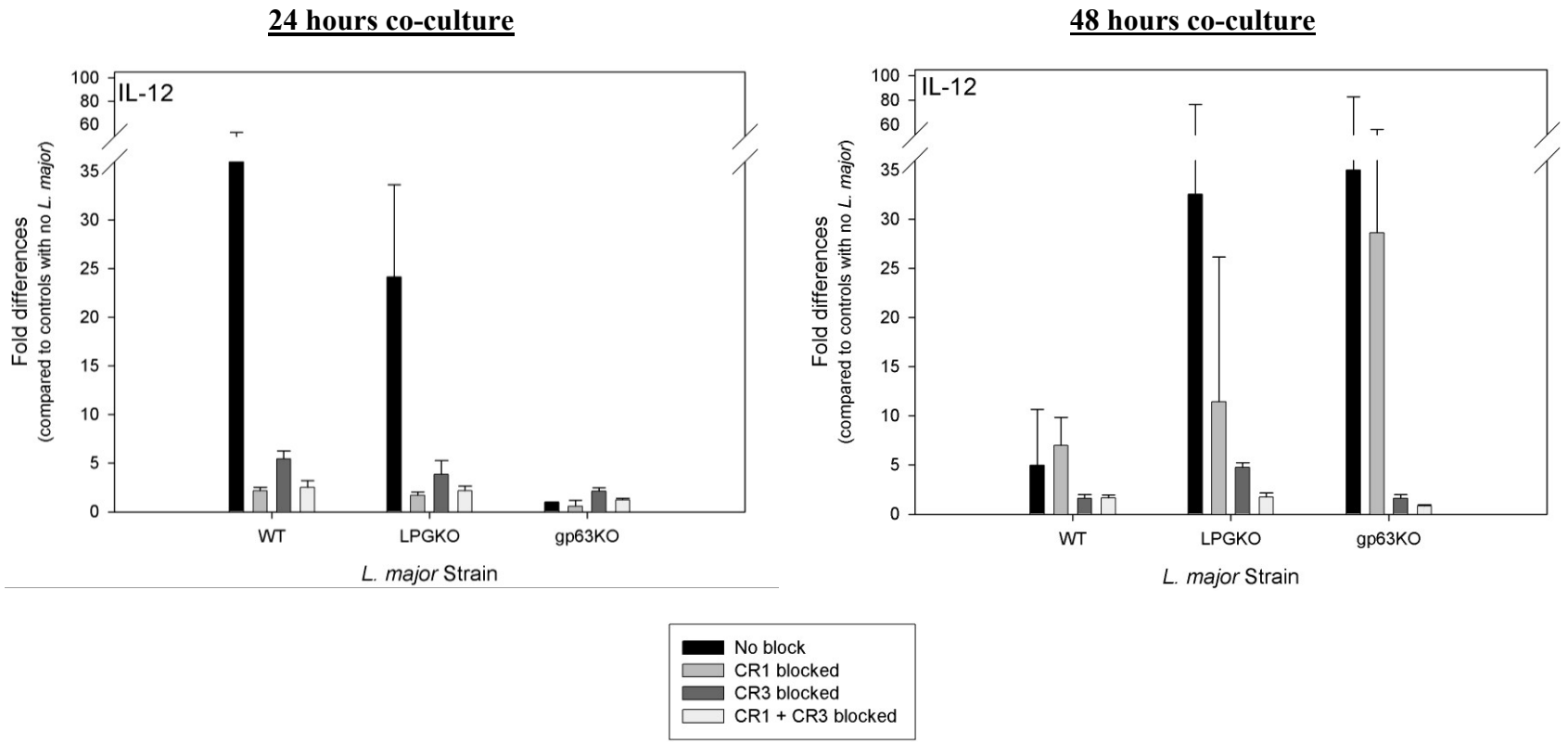

Figure 2.4C – Levels of IL-12 produced by cocultures of naïve, undifferentiated T cells with macrophages infected by *Leishmania major* of wild type (WT), LPGKO or gp63KO strains, with or without blocking of complement receptors (CR1, CR3, CR1+CR3). Cytokine production is expressed in fold differences compared to those produced by similarly blocked or unblocked co-cultures that were not exposed to *L. major*. Macrophages and T-cells were co-cultured for either 24 hours or 48 hours following macrophage blocking by anti-CR antibodies and 5 days of infection by *L. major*.

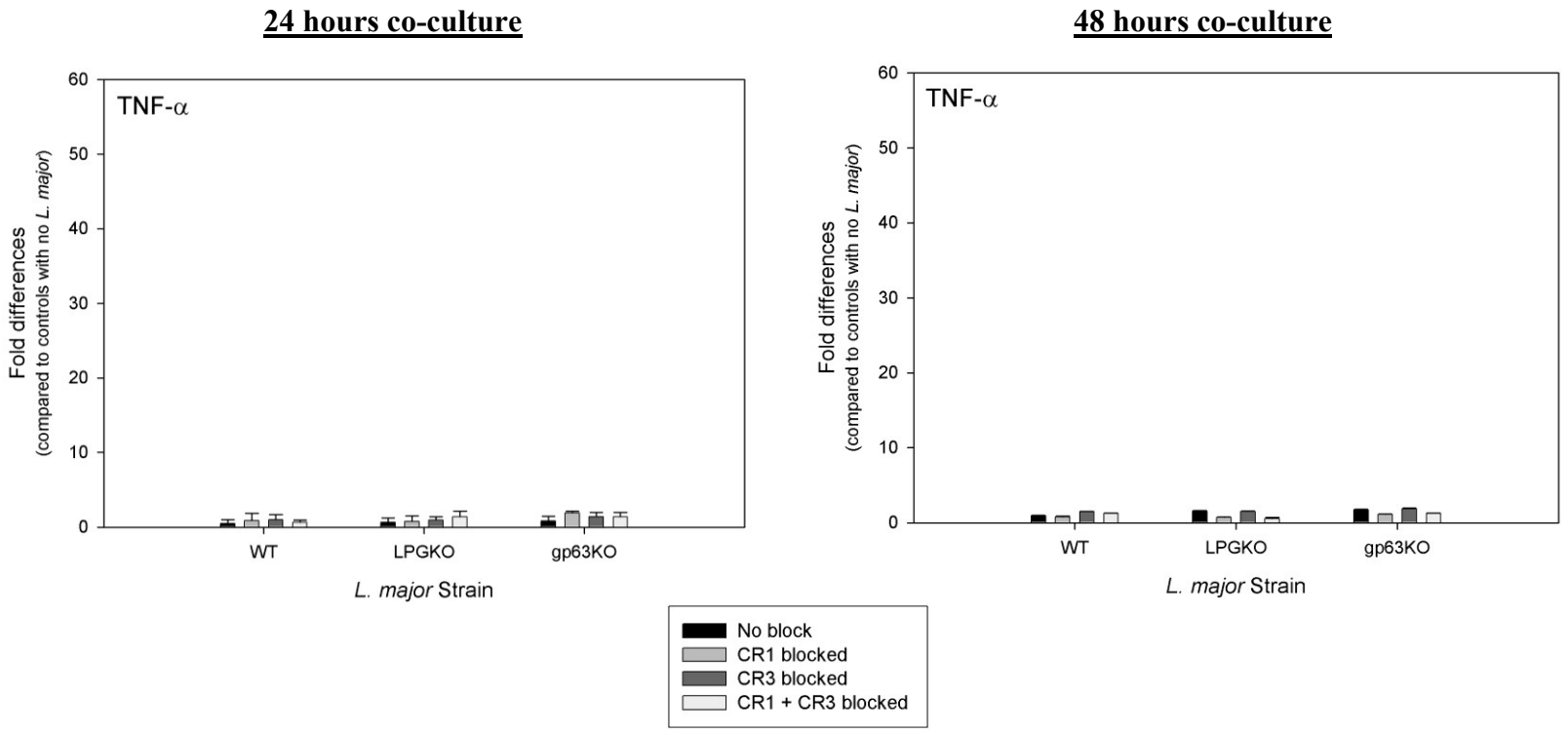

Figure 2.4D – Levels of TNF-α produced by cocultures of naïve, undifferentiated T cells with macrophages infected by *Leishmania major* of wild type (WT), LPGKO or gp63KO strains, with or without blocking of complement receptors (CR1, CR3, CR1+CR3). Cytokine production is expressed in fold differences compared to those produced by similarly blocked or unblocked co-cultures that were not exposed to *L. major*. Macrophages and T-cells were co-cultured for either 24 hours or 48 hours following macrophage blocking by anti-CR antibodies and 5 days of infection by *L. major*.

DISCUSSION

In several experiments, we compared the response between unblocked macrophages and macrophages with CR1 or CR1/2 blocked, CR3 blocked, and combined blocking of CR1 and CR3 when exposed to *L. major* of the wild-type LV39 or A2 strains; the LPGKO strain, lacking key elements of the LPG surface molecule; and the gp63KO strain, which lacks gp63. Phagocytosis was assessed and quantified for varying degrees of infection by all of these strains. Cytokines assessed were chosen based on those involved in Th1/Th2 differentiation and early macrophage response to *L. major* infection. Previous investigations have demonstrated a role for complement receptors in recognition and phagocytosis of *L. major*. Murine complement receptor 1 and 2 (CR1/2) are co-localized and affect to a minor degree the phagocytosis of wild-type (WT) *L. major*, as does blocking of only complement receptor 1 (CR1) (da Silva et al., 1989; Rosenthal et al., 1996). Complement receptor 3 (CR3) is a known receptor for *L. major* phagocytosis and macrophages with CR3 blocked have been shown to have reduced phagocytosis of *L. major* promastigotes and amastigotes (Mosser and Edelson, 1985; Wilson and Pearson, 1988; Puentes et al., 1988).

Phagocytosis of *L. major* of each of the various strains was affected by both strain and complement receptor blocking (Figure 2.2). Decreased phagocytosis of WT *L. major* with blocking of complement receptors has been reported previously, and our results are similar to those seen by others (Wilson and Pearson, 1988; da Silva et al., 1989). The drop in phagocytosis seen with blocking of CR1 demonstrates that CR1 and CR3 are, to a great degree, similar in terms of their importance for *Leishmania* phagocytosis. Neither are necessary for phagocytosis, but are important, with a notable reduction in

phagocytosis of WT *L. major* seen when macrophages were blocked by either CR1 (~50% reduction in infection rate –Figure 2.2) or CR3 (~65% reduction in infection rate – Figure 2.2). Blocking of both did not have a pronounced additive effect on the infection rate with WT parasites (compared to CR3-blocking – Figure 2.2), suggesting that other macrophage surface receptors (fibronectin receptor, toll-like receptors, mannose-fucose receptor, scavenger receptor, etc.) may have at least a small degree of influence on phagocytosis of *L. major* (Mosser and Rosenthal, 1993). The fibronectin receptor has certainly been implicated in a small degree of increased phagocytosis (Chang et al., 1990).

Continuing the above experiments, we assessed the interaction of the *L. major* surface molecules LPG and gp63 with complement receptors. LPG is a known ligand of CR3 and CR1, as well as a site of C3b opsonization. Gp63 is a known ligand of CR3 (Mosser and Edelson, 1985; Mosser and Brittingham, 1997). Little is known about the downstream cytokine responses seen resulting from the interaction between these two surface molecules and macrophage CRs. Parasites lacking in LPG and GIPL (Späth et al., 2000) and gp63 and GIPL (Joshi et al., 1998; Joshi et al., 2002) have been produced and were used in *in vitro* experiments with unblocked and CR-blocked macrophages. Utilizing LPGKO or Gp63KO parasites resulted in generally decreased phagocytosis of parasites and thus macrophage infection rate compared to WT parasites utilizing similar CR-blocking (including unblocked macrophages), except for CR3-blocked macrophages with gp63KO parasites, which had similar infection rates to unblocked gp63KO-infected macrophages (Figure 2.2). This confirms the importance of CR3 interaction with gp63 (Russell and Wright, 1988; Wilson and Pearson, 1988), in that WT parasites with intact

gp63 lose infectivity when CR3 is blocked, while gp63KO parasites do not. Intriguingly, CR1 blocking in LPGKO parasite infection had no significant effects on parasite phagocytosis compared to unblocked LPGKO-infected macrophages as well, which may reinforce the association of LPG with CR1 in phagocytosis (da Silva et al., 1989), rather than CR3, as has been suggested previously (Talamas-Rohana et al., 1990). With the known low infectivity of LPGKO *L. major* in vivo (Späth et al., 2000; Späth et al., 2003), it is unsurprising that these parasites had decreased infection rates in macrophages compared to WT or gp63KO parasites. The observed low rate of infection may instead be a low survival rate, with phagocytosed parasites being destroyed by the macrophages, rather than evading intracellular killing. Given that heat inactivated FBS was used for the media, without active complement components or immunoglobulins, these findings also confirm that LPG and gp63 are directly associated with phagocytosis and macrophage receptor binding, rather than merely as C3b or iC3b binding sites, as suggested previously (Mosser et al., 1992).

Despite blocking of multiple complement receptors and utilization of parasites lacking gp63 or LPG, *L. major* parasites were still able to successfully bind to and infect macrophages, albeit at reduced infection rates. The reduced infection rates indicate the importance of the interaction between gp63 and CR3 and LPG and CR1 for phagocytosis of *L. major*. However, the persistence of infection provides evidence to suggest that LPG and gp63 are not the sole ligands for binding to macrophages and CR1 and CR3 are not the sole receptors (Bosetto et al., 2007). Kedzierski et al. (2004) identified a leucine-rich repeat motif in *L. major* membrane proteophosphoglycan and *L. infantum* parasite surface antigen 2 (PSA-2) that facilitated attachment and phagocytosis through CR3 in mice.

The Fc receptor has been shown to participate in recognition of and phagocytosis o*f L. major*, although through opsonization, rather than directly via a parasite surface motif (Woelbing et al., 2006). Toll-like receptor 2 has been linked with recognition of LPG and may recognize other parasite surface motifs, as do TLR-4 and TLR-9 (de Veer et al., 2003; Kropf et al., 2004; Li et al., 2004; Flandin et al., 2006; Tuon et al., 2008).

IL-6 derived from infected macrophages was slightly elevated in CR1 and CR1+CR3 blocked groups for all strains compared to unblocked groups at 48 hours and only elevated in CR1-blocked groups over unblocked controls at 72 hours (Figure 2.3A), thus suggesting that there was some slight degree of increased production of IL-6 by macrophages when CR1-based recognition of *L. major* was circumvented. However, for co-culture experiments, when CR3 alone or CR1+CR3 were blocked, IL-6 levels from WT-infected and LPGKO-infected groups increased, particularly at the 48 hour timepoint. The elevation in IL-6 levels seen in the co-culture experiments, particularly at the 48 hour timepoint (Table 2.2A and Figure 2.4A), suggests that IL-6 is primarily being produced by T-cells following stimulation by infected macrophages during co-culture. However, both gp63 and LPG do appear to play a role in stimulation of IL-6 production, given the generally decreased levels of IL-6 produced by both macrophages and co-cultured T-cells when infected by *L. major* with those ligands deleted. Similarly, it would appear as though CR1 and CR3 may both have some influence on the ability of *L. major* to silently infect macrophages, as blocking these CR produced increased levels of IL-6, particularly in co-culture experiments (Table 2.2A and Figure 2.4A). IL-6 is an important pro-inflammatory cytokine, with some degree of influence on the differentiation of naïve CD4+ T-cells, capable of influencing differentiation towards a

Th2 phenotype, Th17 phenotype (in conjunction with TGFβ), or even another Treg cell phenotype altogether (Dienz and Rincon, 2009). There is ample evidence in the literature for IL-6 controlling numerous other cytokines, including both Th1- and Th2-associated cytokines. Rincon et al. (1997) showed IL-6 has the ability to direct the differentiation of CD4+ T cells to become IL-4-producing, potentially thus biasing the T cell response towards Th2, although this was not seen in our experiments. This has not been borne out as an important factor in *in vivo* experiments with IL-6-deficient mice, as those mice prove to remain resistant to *L. major*, rather than become susceptible (Moskowitz et al., 1997). However, susceptible BALB/c mice lacking in IL-6 do not change their susceptible phenotype and have alterations in both Th1 and Th2 cytokine mRNA levels, specifically reduction in IL-4, IL-10, IL-12, and IL-13 levels (Titus et al., 2001). The elevation of IL-10 seen in these experiments could be associated with the elevation of IL-6, as IL-6 has been known to influence IL-10 production and may also downregulate IL-12 production as a result (Dienz and Rincon, 2009).

IL-10 plays an important role in promotion of a Th2 response, suppression of Th1 responses and exacerbation of infection (Moore et al., 2001). While this is more commonly associated with IL-4 in experimental *Leishmania* infection, IL-10 has a notable effect on macrophage activation and response to *Leishmania*. IL-10 can inhibit macrophage activation by making macrophages refractory to IFN-γ stimulation, inducing a Th2-type response (Kane and Mosser, 2001). Fc receptor-mediated uptake of *L. major* has been shown to induce IL-10 production in dendritic cells (von Stebut, 2007) and increased availability of *L. major* to the Fc receptor or other receptors on macrophages may have influenced the increased IL-10 production seen with CR1, CR3, and CR1+CR3

blocking in WT parasites and CR3 and CR1+CR3 blocking in LPGKO and gp63KO parasites at 48 hours, although this does not explain the dramatic increase seen in IL-10 at 72 hours from the unblocked macrophages infected with all three *L. major* strains (Table 2.1A and Figure 2.3B). IL-10 levels were only slightly altered by CR-blocking or differences in *L. major* strain in the co-cultured experiments (Table 2.2A and Figure 2.4B), perhaps due to an inability of the *Leishmania*-affected macrophages to induce Th1/Th2 differentiation. The early induction of IL-10 seen in these experiments (Table 2.1A and Figure 2.3B) may have some long lasting suppression of Th1 differentiation in the co-culture experiments.

IL-12 is a key cytokine in the generation of a protective Th1 response to *L. major* infection. IL-12 is also a key factor in the induction of IFN-γ (Trinchieri, 2003). *Leishmania* promastigotes have been shown to selectively inhibit IL-12 induction in infected macrophages (Carrera et al., 1996). This downregulation of IL-12 could lead to a failure in macrophage activation, with reduced IFN-γ and NO production, and subsequent failure to kill intracellular *Leishmania*. Intriguingly, IL-12 levels were high in the infected macrophages, generally increasing from 24 to 48 hours post infection, but dramatically reduced in the co-cultured supernatants. This may be due to the increased timecourse of the co-culture experiments, coupled with the reported inability of *Leishmania*-infected macrophages to induce IL-12 production in naïve CD4+ T-cells (Woelbing et al., 2006), unlike dendritic cells (Konecny et al., 1999). As these T-cells were not primed with IFN-γ and/or LPS, it may have been impossible for the infected macrophages to induce their Th1 differentiation and production of IL-12 in our co-culture experiments. Blocking of the complement receptors resulted in decreased IL-12

production compared to unblocked macrophages, with an additive effect of blocking both CR1 and CR3 (resulting in greater decreases) (Table 2.1B and Figure 2.3C). This pattern was generally seen in the co-cultured experiments as well, albeit primarily in the CR-blocked WT and LPGKO *L. major*, with only the 48 hour co-culture of gp63KO-infected macrophages similarly affected (Table 2.2B and Figure 2.4C). CR3 ligation has been associated with inhibition of IL-12 production (Kwan et al., 1992). Additionally, in CD11b-deficient BALB/c mice, the resultant CR3-deficiency has been associated with resistance to *L. major* infection in vivo, despite having no reported alterations in phagocytosis or cytokine production in vitro (Carter et al., 2009), unlike those seen in our experiments. Thus, it would seem that the ligation of CR3 by the anti-CR3 antibody is a major inciting element in the decreased IL-12 production seen here, although this does not explain the observed decrease in IL-12 from blocking CR1 in the macrophage infection or co-culture experiments (Tables 2.1B and 2.2B, Figures 2.3C and 2.4C). The increased IL-12 levels seen in the LPGKO infected macrophages do not appear to be due to the activation of receptors other than CR3 upon macrophage phagocytosis, as this is not seen in the gp63KO parasites, which generally show a very robust link between CR3 and gp63. Perhaps LPG has a degree of IL-12 inhibitory activity and the LPGKO parasites lack this inhibition, resulting in the increased IL-12 levels seen here (Tables 2.1B and 2.2B, Figures 2.3C and 2.4C).

TNF-α is another of the cytokines that plays a role in the generation of a protective Th1 response to *Leishmania* infection. The initial TNF-α levels seen in CR1 and CR3-blocked macrophages were robust in all three strains of *L. major*, and significantly increased over those seen in unblocked macrophages, with CR1 levels

higher than CR3. This went away at the later 72 hour timepoint (Table 2.1B and Figure 2.3D). In the co-culture experiments, the TNF-α response was slight and did not follow the initial pattern seen in the macrophage-only experiment (Table 2.2B and Figure 2.4D). The failure of the TNF-α response to develop or progress in the co-culture experiments adds more evidence to the lack of any Th1 differentiation of the naïve T-cells and of development of a protective response. The initial high levels of TNF-α found in the macrophage-only experiments certainly shows that there was a strong response to the parasites at initial exposure. This may have gone away without some form of co-stimulation or other continued stimulation, as parasites were phagocytosed and processed by the macrophages. Alternatively, the increased levels of IL-6 and IL-10 seen at the 72 hour timepoint may have suppressed TNF-α production to a degree.

Interestingly, there was no evidence of any significant effect on IL-4, IFN-γ, or NO levels in any of the experiments. These are cytokines that are directly linked to Th1 (IFN-γ and NO) or Th2 (IL-4) differentiation or are primarily involved in the leishmanicidal response by macrophages (NO). NO and IFN-γ are inter-related, with inducible nitric oxide synthetase (iNOS)-derived NO serving as a cofactor for the induction of IFN-γ production and IFN-γ serving as one possible inducer of iNOS (Dalton et al., 1993; Dieffenbach et al., 1999; Huang et al., 1993). Thus, the absence of an IFN-γ response may be one reason why there was not a detectable and ongoing NO response. Additionally, the low IL-12 response seen in the CR-blocked groups would contribute to the low IFN-γ and NO levels, although that does not explain the lack of a response in the unblocked macrophages. However, it would appear that the lack of naïve

CD4+ T-cell activation by the infected macrophages played the most important role in both the absence of IFN-γ and IL-4 in these experiments.

The findings seen in this *in vitro* study suggest that the role of both complement receptors and parasite surface molecules gp63 and LPG in dictating Th1/Th2 bias is limited at best and possibly functionally non-existent, despite their demonstrated and reported effects on phagocytosis of *L. major*. Perhaps the stimuli offered by a single *in vitro* exposure to *Leishmania* is not sufficient to induce a strong Th1/Th2 bias or perhaps that the in vitro milieu was not conducive to differentiation of the naïve CD4+ T-cells. This is suggested in the work of Woelbing et al. (2006), which showed antigen presentation by macrophages infected with *L. major* through CR3-mediated phagocytosis only occurred to primed, rather than naïve, T cells. Alternatively, the response was too limited to be detected by our methods, although that seems to be unlikely. Another possibility may be that the effects are not readily seen in an *in vitro* setting, as these are complex interactions between the parasite and multiple host cell types, cytokines, and cell-surface receptors. The most probable likelihood is that the stimulus for Th1/Th2 differentiation was insufficient, either because macrophages are inadequate stimulators of Th1/Th2 differentiation (dendritic cells are the primary cells involved in this) or because there were multiple contradictory signals involved in affecting the Th1/Th2 bias, including IL-6 & IL-10 (Th2) and TNF-α and IL-12 (Th1). In light of these possibilities, *in vivo* experiments assessing the infectivity of these strains of *L. major* and host response in strains of resistant and susceptible mice may be the most biologically relevant method of assessing these interactions.

CHAPTER 3

NANODISK-ASSOCIATED AMPHOTERICIN B CLEARS *LEISHMANIA MAJOR* CUTANEOUS INFECTION IN SUSCEPTIBLE BALB/C MICE

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CHAPTER SYNOPSIS

Nanometer scale, apolipoprotein-stabilized phospholipid bilayer disk complexes (nanodisk; ND) harboring the toxic and poorly soluble antileishmanial agent, amphotericin B (AMB), were examined for efficacy in treatment of *Leishmania major* infected BALB/c mice (*Mus musculus*). *L. major* infected mice were intra-peritoneally (IP) treated with AMB-ND in 0, 1 and 5 mg/kg doses at 24 h, 48 h, 4, 7, 14, and 21 days post-infection in two experiments. *L. major* infected mice were IP treated with PBS, 5 mg/kg AMB-ND or 5 mg/kg lipid-associated amphotericin B (liposomal amphotericin B, AmBisome™) at 24 h, 48 h, 10, 20, 30 and 40 days post-infection in one experiment. Parasite numbers, footpad lesion size progression and development of cytokine responses were assayed at days 7, 15, 30, 50, 140, and 250 or days 14, 30, 50, 95, and 140 post-infection. Mice administered AMB-ND in 1 or 5 mg/kg doses were significantly protected from *L. major*, displaying decreases in lesion size and parasite burden, particularly at the 5 mg/kg dosage level. In contrast to the IP-treated AmBisome group, BALB/c mice treated with IP AMB-ND completely clear an *L. major* infection by 140-250 days post-infection, with no lesions remaining and no parasites isolated from infected animals. Restimulated mixed lymphocyte culture cytokine responses (IL-4, IL-12, IL-10, NO, IFN-γ) were unchanged by AMB-ND administration compared to controls. The marked clearance of *Leishmania* parasites from a susceptible strain of mice without an appreciable change in the cytokine response suggests that AMB-ND represent a potentially useful formulation for treatment of intra-histiocytic organisms.

INTRODUCTION

Leishmaniasis is a tropical disease caused by a genus of protozoal parasites, *Leishmania* spp. Infection with *Leishmania* spp. will cause disease ranging from single cutaneous ulcers to life-threatening hepatomegaly and splenomegaly. With its emergence as an opportunistic infection in HIV positive individuals, effective treatment of leishmaniasis is of increased importance (Murray, 1999). Currently, there are no vaccine protocols for leishmaniasis and many therapies are limited by toxicity, long-term courses of treatment, expense and development of parasite resistance to chemotherapeutics (Singh and Sivakumar, 2004). Currently used drugs include pentavalent antimonials such as sodium stibogluconate (Pentostam™), different amphotericin B (AMB) lipid formulations, and other, lesser used drugs, such as pentamidine, allopurinol, miltefosine, and ketoconazole (Singh and Sivakumar, 2004; Hepburn, 2003; Croft and Coombs, 2003). With all of these agents varying degrees of toxicity are seen, and full clearance of parasites is not always achieved in cutaneous leishmaniasis, both in clinical practice and in experimental studies (Singh and Sivakumar, 2004; Sundar, 2001). As a method of countering these limitations, efforts have been made both to develop new drugs and improved methods of delivering known anti-protozoal drugs without concomitant toxicity to the human host. Of particular interest is the possibility of targeting therapeutics to the macrophage phagolysosome, the site of *Leishmania* amastigote reproduction (Basu and Lala, 2004; Croft and Coombs, 2003; Datta et al., 2003; Demidova and Hamblin, 2004; Medda et al., 2003; Nan et al., 2004; Singh and Sivakumar, 2004; Tempone et al., 2004). Such a strategy would conceivably reduce the required treatment dose and, thereby, associated toxicity, expense, and treatment duration.

Recently, methods for the generation of AMB-enriched reconstituted high-density lipoprotein particles have been described (Oda et al., 2006). The particles generated are comprised of a nanometer scale, disk-shaped phospholipid bilayer, hereafter referred to as nanodisks (ND), whose periphery is circumscribed by amphipathic apolipoprotein molecules. Apolipoproteins are well known plasma components that function in transport of hydrophobic biomolecules, including cholesterol, triacylglycerol, phospholipid and fat-soluble vitamins. A common property shared by these proteins is an ability to disrupt certain phospholipid bilayer vesicles and transform them into disc-shaped lipid/protein complexes (Raussens et al., 1998; Narayanaswami et al., 2004). Hydrophobic drugs such as the polyene antibiotic, AMB, can intercalate between phospholipids in the bilayer component of the complex, effectively solubilizing the active biomolecule. NDs are distinguished from conventional liposomes or lipid microvesicles in that they do not possess an aqueous core, they are fully soluble in aqueous media, their diameters range from 8-20 nm (rather than 60-250 nm for liposomes), and apolipoproteins are an intrinsic structural element of the complex (Narayanaswami et al., 2004).

In the susceptible BALB/c mouse model, there is evidence that conventional drug therapies alone are often insufficient for clearance of cutaneous *Leishmania* infection. Nabors et al. (1995) and Li et al. (1997) showed that clearance of established cutaneous *L. major* infections in BALB/c mice required not only Pentostam™ (sodium stibogluconate) but also therapy with IL-12 or IFN-γ to up-regulate IL-12 levels and trend the animals towards a Th1 immune response. Similar necessity for IL-12 has been shown by other researchers (Murray et al., 2000b). In short, in the murine model,

successful treatment of cutaneous leishmaniasis with pentavalent antimonials must often be accompanied by an immune response similar to that seen in a resistant animal to achieve full clearance of infection. AMB therapy may also require an immune response, especially tumor necrosis factor (Murray et al., 2000a). Other researchers, however, have claimed that AMB can act independent of an immune response (Murray et al., 2000b; Escobar et al., 2001). There is recent evidence that AMB derivatives may negatively influence B-cell responses, as well as increase TNF-α production (Ehrenfreund-Kleinman et al., 2005).

In this study, we determined the efficacy of AMB-ND complexes in treatment of *L. major* cutaneous infection. The data demonstrate that six 5 mg/kg doses of AMB-ND delivered at 1 -10 day intervals over the course of three to 5 weeks are capable of clearing a *L. major* infection in the BALB/c mouse. This novel lipid formulation of AMB is significantly more efficacious for treatment of cutaneous leishmaniasis than similar doses of the liposomal AMB formulation, AmBisome™, and resulted in parasite clearance without statistically significant changes in immune response compared to controls.

MATERIALS AND METHODS

Mice

Adult female BALB/c mice (6 - 10 week old) were procured from the National Cancer Institute. Mice were kept in colonies at Colorado State University, under supervision of the CSU Laboratory Animal Resources Department with authorization by the Animal Care and Use Committee. Mice were anesthetized with IP Ketamine (75 mg/kg) and Xylazine (15 mg/kg) prior to subcutaneous inoculation with 1 x 10^6 *L. major* parasites in 50μL of DMEM in the left hind footpad. Mice were treated with AMB-ND or AmBisome (Astellas Pharma US, Deerfield, IL) at concentrations of 1 mg/kg or 5 mg/kg AMB in 200μL total volume/mouse. Control mice were injected with an equivalent amount of empty ND, lacking AMB, in the first two experiments and PBS the final experiment. Mice were injected in the peritoneal cavity at each treatment time point. Treatment time points for the first experiments (0, 1 and 5 mg/kg AMB-ND) were 24 h, 48 h, 7 days, 14 days, and 21 days post-infection. The final experiment, with PBS (Control), AmBisome (5mg/kg AMB) or AMB-ND (5 mg/kg AMB) groups, had treatments at 24 h, 48 h, 10 days, 20 days, 30 days, and 40 days post-infection. Footpad lesion size was measured using a Vernier caliper at 2 - 4 day intervals throughout a given experiment. For measurement of lesion size the infected left footpad was compared to the normal right footpad. Mice were euthanized in a CO_2 chamber at day 7, day 15, day 30, day 50, and indicated later time points, to assess parasite burden and cytokine responses of re-stimulated lymph node cells.

Parasites

L. major parasites from the LV39 strain were grown on sheep blood agar in parasite growth medium [RPMI-1640 (Sigma-Aldrich, St. Louis, MO) with 5% fetal bovine serum (Hyclone, Logan, UT), 10mM Hepes (Sigma-Aldrich), 100U/mL penicillin (Gibco, Carlsbad, CA), 100μg/mL streptomycin (Gibco), 2mM L-glutamine (Gibco), 1mM sodium pyruvate (Gibco), 0.2mM L-asparagine (Calbiochem, San Diego, CA), 0.6mM L-arginine (Calbiochem), and 2% sterile-filtered normal human urine]. Parasites were passaged through mice every 2 months to retain virulence and from flask to flask twice a week. At 5 - 6 days after flask inoculation, parasites were removed from the growth medium, centrifuged to remove dead parasites, washed in DMEM and counted on a Neubauer hematocytometer prior to re-suspending in DMEM.

Nanodisks

AMB-ND and empty ND were produced essentially as described by Oda et al. (2006). Briefly, ten mg of dispersed phospholipid vesicle substrate comprised of 7 mg dimyristoylphosphatidylcholine and 3 mg dimyristoylphosphatidylglycerol plus 2.5 mg AMB were incubated with 4 mg recombinant human apolipoprotein A-I at 24 °C. Following nanodisk formation and dialysis against PBS, ND associated AMB was determined spectrophotometrically at 416 nm by dissolving an aliquot of the ND solution in dimethylsulfoxide (extinction coefficient = 1.214×10^5 M^{-1} cm^{-1}). Sterile filtered NDs were stored in the dark at 4 °C for < 40 days. ND preparations were diluted in phosphate buffered saline (PBS), pH 7.0, to the respective concentrations for each treatment group.

Parasite Quantification

Parasite burden was evaluated using a limiting dilution assay. Footpads were skinned and removed from euthanized mice. Footpads from multiple mice from the same group were ground in a tissue homogenizer in modified Schneider's Insect Medium (Sigma-Aldrich) containing 10% fetal bovine serum, 10mM Hepes, 100U/mL penicillin, 100μg/mL streptomycin, 50μg/mL gentamicin (Sigma-Aldrich), 2mM L-glutamine, 1mM sodium pyruvate, 0.2mM L-asparagine, 0.6mM L-arginine, and 2% sterile-filtered normal human urine. Pooled samples were diluted in 5ml modified Schneider's Insect Medium per infected footpad. Samples were plated in 100μL aliquots on 96 well plates, with a 10-fold dilution of each row. Plates were sealed and set in the dark at room temperature for 10 - 14 days before evaluation. Plates were scored and the results evaluated using the ELIDA program (ELIDA Software© 1985-2005, Carl Taswell) for statistical analysis (Taswell, 1987).

Cytokine Assays

Popliteal and inguinal lymph nodes draining the infected footpads were removed from euthanized mice. Lymph nodes from multiple mice in the same treatment groups were pooled. Lymph nodes were placed in Dulbecco's modified Eagle Medium (DMEM; Gibco) and squashed using sterile metal screens to release cells. Mixed cell populations isolated from the lymph nodes were washed and pelleted 3 times in DMEM at 1000 rpm. Resuspended cells were mixed 1:1 with Trypan Blue stain (Invitrogen) and counted on a Neubauer hematocytometer. Counted live cells were diluted to 5 x 10^6 cells/mL in DMEM cell media [DMEM, 10mM Hepes, 100U/mL penicillin, 100μg/mL streptomycin, 2mM L-glutamine, 1mM sodium pyruvate, 0.2mM L-asparagine, 0.6mM

L-arginine and 0.5% normal mouse serum] and added to 24 well plates at 1mL/well. *L. major* promastigotes were added to the plates at 2 x 10^6 parasites/well. Lymph node cells were cultured at 37 °C in 5% CO_2 for 48 h at which time supernatants were collected and stored at ⁻20 °C. Commercially available ELISA's were performed to determine levels of IFN-γ, IL-12, IL-4, and IL-10 (BD Pharmingen, San Jose, CA) in supernatants. Samples were assayed for levels of NO utilizing the Griess reaction (Sigma).

Anti-Leishmania Antibody Assay

L. major promastigotes were washed in 1 x PBS, resuspended at a concentration of 1 x 10^8 parasites/mL and sheared with a 30g needle. LDS loading dye (Invitrogen, Carlsbad, CA) was added and the lysates stored at ⁻20 °C. Aliquots of the *L. major* lysate (10 μL) were heated at 95 °C for 5 min, cooled and loaded into lanes of a 4-12% Bis/Tris polyacrylamide gel (Invitrogen) and separated by electrophoresis in 1 x MOPS buffer under reducing conditions. The gel was transferred to nitrocellulose in Towbin buffer (10% methanol, 25 mM Tris-HCl, 192 mM glycine) and blocked in 1% gelatin. The nitrocellulose was tested for completeness of protein transfer with MemCode (Pierce, Rockford, IL). Nitrocellulose strips were incubated for 1 h with a given mouse serum diluted 1:100 in 50 mM Tris-HCl pH 7.5, 150 mM sodium chloride, 0.1 % Tween 20 (TNT). Blood from different *L. major* infected BALB/c mice was collected by cardiac puncture at euthanasia 50 days following inoculation with 1 x 10^6 LV39 *L. major* promastigotes and subsequent treatment with PBS (Control), AmBisome (3 mg/kg or 5mg/kg AMB) or AMB-ND (3 mg/kg or 5 mg/kg AMB). Blood from mice in each treatment group was centrifuged to obtain serum, which was stored at ⁻20 °C until use. Following incubation with serum, nitrocellulose strips were washed 3 times with TNT

then reacted with a 1:1000 dilution of alkaline phosphatase-conjugated goat anti mouse IgG in TNT. After multiple washings the nitrocellulose strips were developed with the BCIP/NBT reagent. An identical gel was subjected to silver stain analysis (SilverExpress, Invitrogen) following the supplier's directions.

Statistical analysis

The Student paired *t* test & ANOVA were used for statistical analyses of the footpad lesion size data and the cytokine response data (Systat 9.0). The parasite numbers per footpad was analyzed using the ELIDA software (Taswell, 1987) for initial analysis and, secondarily, ANOVA on linear plots of the data. Differences were considered significant when $p < 0.05$.

RESULTS

Effect of AMB-ND on survival and lesion size in L. major infected mice

Footpad injection of BALB/c mice with *L. major* promastigotes results in the appearance of measurable lesions. In untreated animals or those treated with empty ND lacking AMB, lesion size continues to increase as a function of time, up to day 50, when lesions were judged to be severe and mice were euthanized (**Figure 3.1)**. By contrast, *L. major* infected mice treated with AMB-ND at 1 mg/kg or 5 mg/kg survived a minimum of 100 days longer than empty ND controls ($p < 0.0001$) before developing comparable lesions and being euthanized. Animals in all groups were euthanized if they reached a predetermined level of lesion severity and/or displayed clinical signs of distress or pain. A dose-response was observed between lesion size and AMB-ND treatment with decreased lesion size associated with increasing AMB-ND dose ($p < 0.0001$). Remarkably, animals treated with 5 mg/kg AMB-ND had no measurable footpad lesions by day 150 post infection.

Effect of AMB-ND treatment on L. major parasite numbers

L. major infected mice treated with AMB-ND at concentrations of 1 mg/kg or 5 mg/kg had significantly ($p < 0.05$ from ELIDA (Taswell, 1987)) lower parasite numbers/footpad than untreated controls (**Figure 3.2**). Following cessation of therapy, mice in the 1 mg/kg AMB-ND treatment group eventually developed parasite levels equivalent to those seen in control animals at the time of euthanasia, although this took over 100 days longer. On the other hand, mice treated with 5 mg/kg AMB-ND had parasite numbers/footpad that were progressively lower than those from both the control and 1 mg/kg AMB-ND treatment groups ($p < 0.0001$ from ANOVA). Most significantly,

at the time of the final two determinations of parasite numbers (days 150 and 250), those mice treated with 5 mg/kg AMB-ND had mean parasite numbers/footpad of 0.4, with a 95% confidence interval of 0-1, indicating effective eradication of the parasite from the infection site.

Cytokine responses in L. major infected mice

ELISA assays were performed in triplicate on samples obtained from 2 separate experiments. Cytokine assays of *L. major* infected mice showed no significant differences between the AMB-ND and control treatment groups (**Table 3.1**). Both groups manifest similar cytokine responses, with low IFN-γ, IL-12, and NO production, and moderate to high amounts of IL-4 and IL-10. The only changes evident in the AMB-ND treatment groups were a mild increase in IFN-γ and a slight decrease in IL-4 and IL-10 at later time points (days 50, 140, and 250). However, because no animals in the control, empty ND treatment group survived past day 50, this may be a non-significant change for the later time points, although we cannot assess that.

Effect of lipid formulation on AMB efficacy in L. major infected mice.

To evaluate the extent to which features of the ND particle structure or composition are responsible for the observed efficacy against *L. major* infection in mice, AMB-ND were compared directly with the liposomal AMB formulation, AmBisome, in a single experiment. Using an identical AMB dose and treatment regimen, AMB-ND treated mice displayed decreased lesion size as a function of time and increased survival compared to AmBisome treated mice, which developed severe, life-threatening lesions by day 95 post-infection and were euthanized (**Figure 3.3**). Lesion size was uniformly

statistically significantly different between the AMB-ND and AmBisome treatment groups ($p < 0.0001$). Similarly, AMB-ND treatment groups had significantly lower ($p < 0.05$) parasite numbers/footpad compared to the corresponding AmBisome treatment groups, particularly at later time-points (days 36, 50 and 95) (**Figure 3.4**). A 10-100 fold decrease in parasite numbers was observed for the AMB-ND treatment group compared to the AmBisome treatment group when administered equivalent doses of AMB at similar time points.

Cytokine assays showed no significant differences between cytokine responses for control, AmBisome, or AMB-ND treatment groups (**Table 3.2**).

AMB-ND treatment induces an antibody response to L. major

To assess the humoral immune response to *L. major* and verify infection, AMB-ND and AmBisome treated animals or control animals treated with PBS were assayed for *L. major* antigen reactivity. Although there was recognition of *L. major* antigens by sera from the 5mg/kg AMB-ND group, the profile of proteins recognized was different from that of other serum samples. Also, there was an apparent decreased production of antibody against *L. major* antigens by mice in this treatment group, as evidenced by decreased immunostaining (**Figure 3.5**). In this treatment group, bands seen in other treatment groups at 55, 30, and 17-20 kDa were not present. However, there was reaction against a unique band at ~32 kDa. Treatment with 3 mg/kg AMB-ND, 3 mg/kg AmBisome, or 5 mg/kg AmBisome did not produce any significant differences in antibody responses to *L. major* antigens compared to PBS-treated controls, as judged by Western blot analysis.

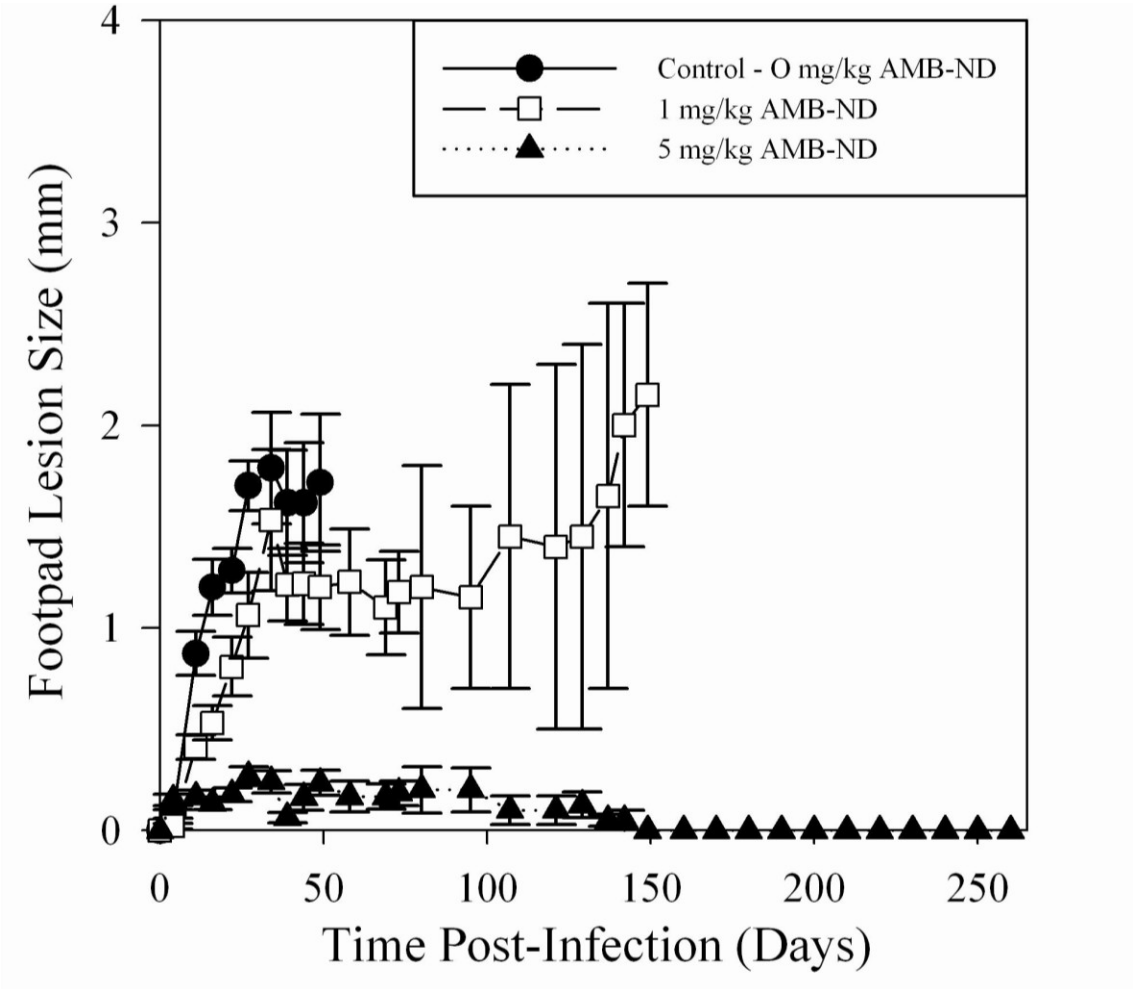

Figure 3.1: Effect of AMB-ND treatment on footpad lesion size in *L. major* infected BALB/c mice. Mice were infected with 1 x 10^6 LV39 *L. major* promastigotes and treated at 24 h, 48 h, 7 days, 14 days, and 21 days post-infection with empty ND or AMB-ND (0, 1 or 5 mg/kg AMB per treatment). Footpad lesion size was measured at the indicated time points. Values plotted are the mean ± standard deviation of 4-20 measurements. Values are averaged from multiple (n=2) experiments utilizing identical parameters.

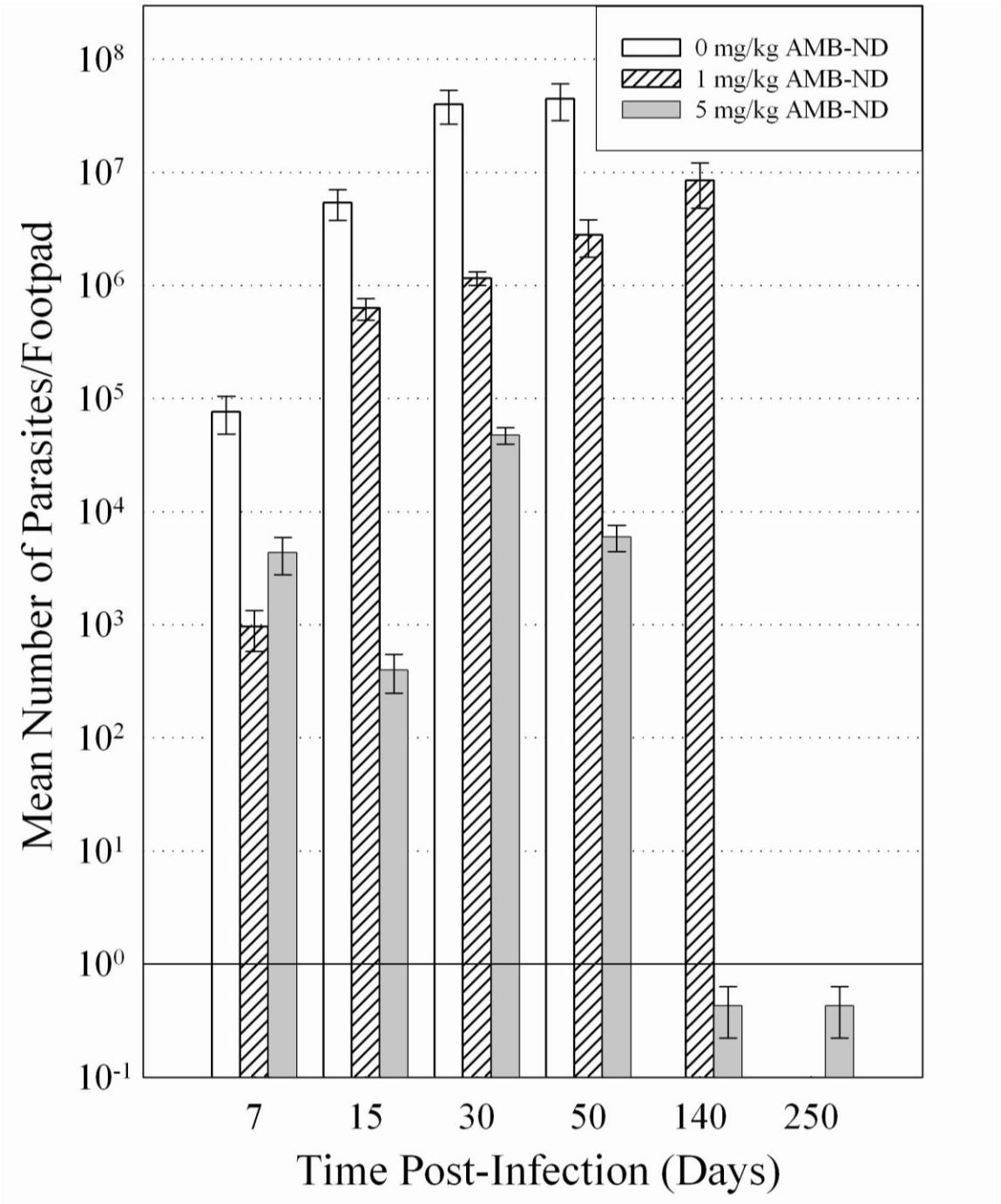

Figure 3.2: Effect of AMB-ND treatment on parasite burden in *L. major* infected BALB/c mice. Mice were infected with 1 x 10^6 LV39 *L. major* promastigotes and treated at 24 h, 48 h, 7 days, 14 days, and 21 days post-infection with empty ND or AMB-ND (0, 1 or 5 mg/kg AMB per treatment). Parasite numbers/footpad were determined as described in Materials and Methods from samples obtained at the indicated time points. Values reported are the mean ± 95 % confidence limits of parasite numbers based on limiting dilution analysis as described in Methods. Values are averaged from multiple (n=2) experiments utilizing identical parameters.

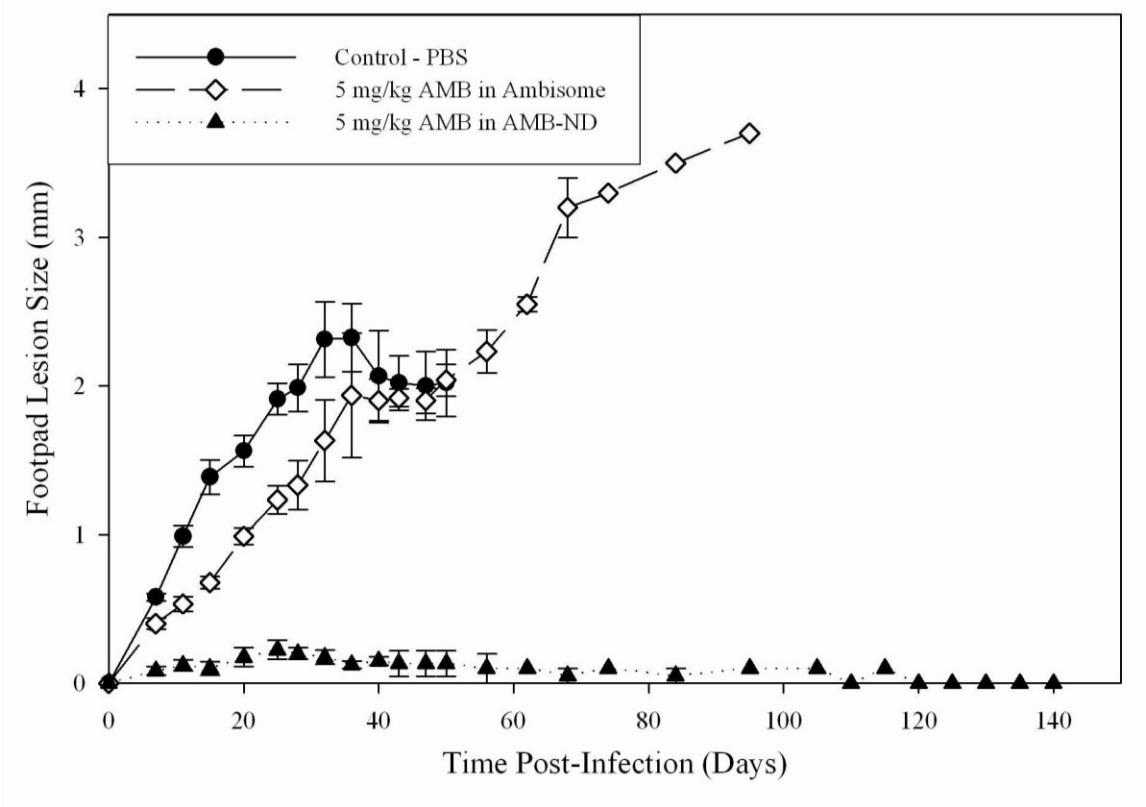

Figure 3.3: Effect of AMB formulation on foot pad lesion size in *L. major* infected BALB/c mice. Groups of mice were infected with 1 x 10^6 LV39 *L. major* promastigotes and treated at 24 h, 48 h, 10 days, 20 days, 30 days, and 40 days with PBS (Control), 5mg/kg AMB in AmBisome, or 5 mg/kg AMB in AMB-ND. Footpad lesion size was measured at the indicated time points. Values plotted are the mean ± standard deviation of 2-14 measurements. Data is derived from a single experiment (n=1).

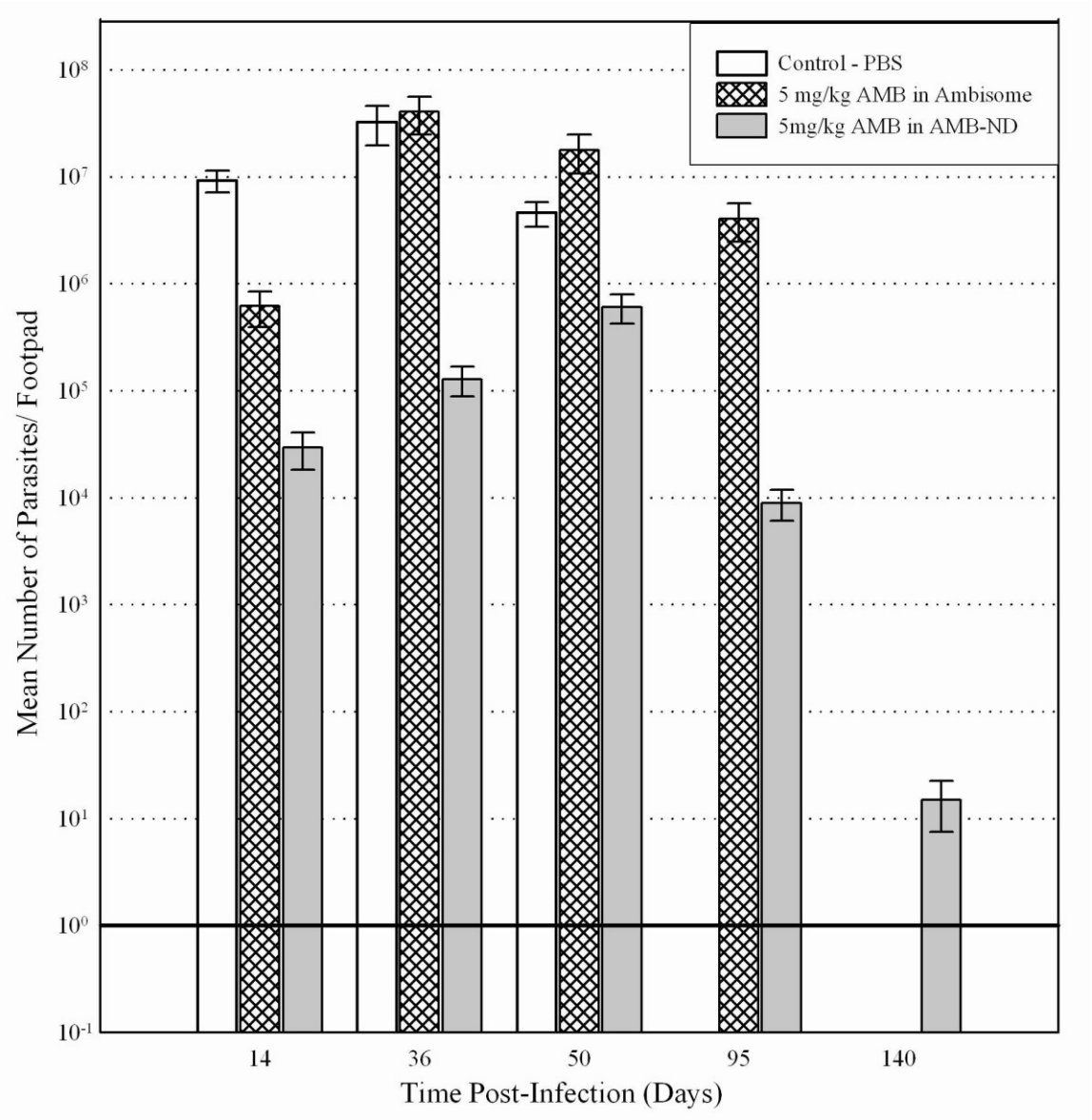

Figure 3.4: Effect of AMB formulation on parasite numbers/footpad in *L. major* infected BALB/c mice. Groups of mice were infected with 1 x 10^6 LV39 *L. major* promastigotes and treated at 24 h, 48 h, 10 days, 20 days, 30 days, and 40 days with PBS (Control), 5mg/kg AMB in AmBisome, or 5 mg/kg AMB in AMB-ND. Parasite numbers/footpad were determined as described in Materials and Methods from samples obtained at the indicated time points. Values reported are the mean ± 95 % confidence limits of parasite numbers based on limiting dilution analysis as described in Material and Methods. Data is derived from a single experiment (n=1).

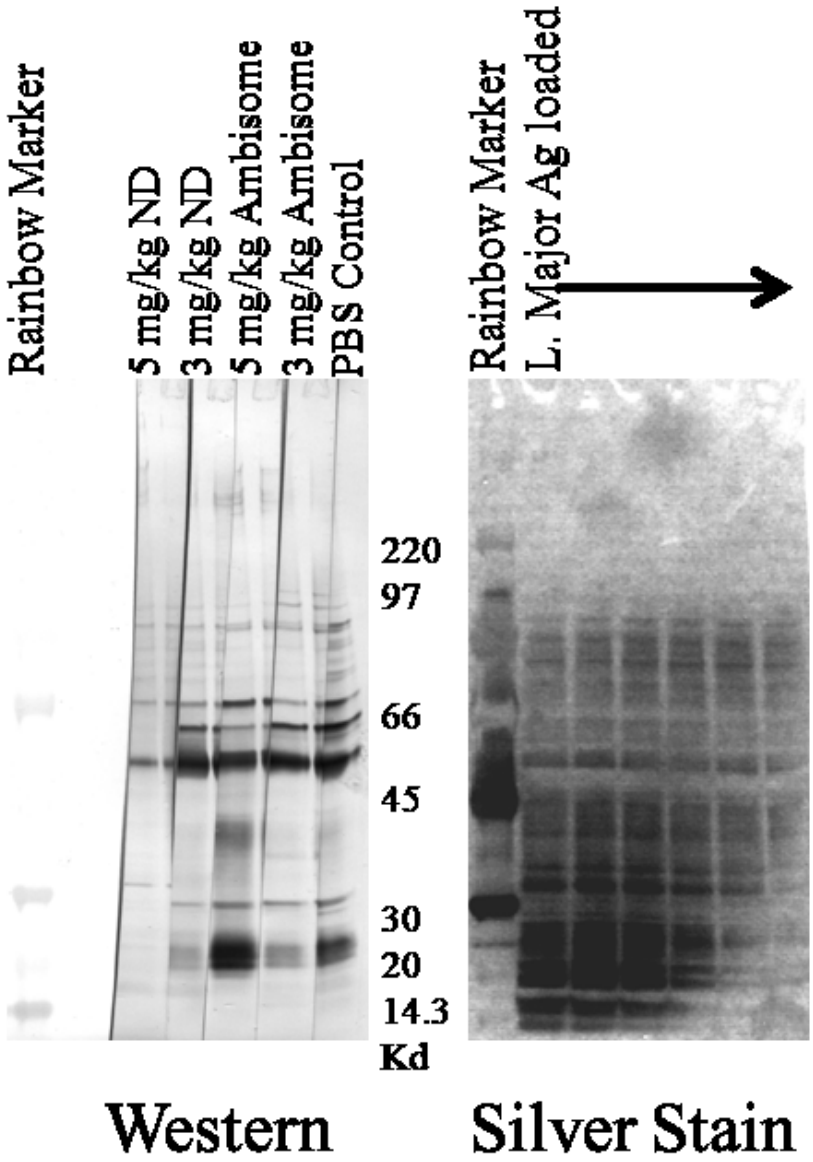

Figure 3.5: Immunoblot of *L. major* antigens. *L. major* promastigote lysate proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The membrane was probed with anti-*L. major* BALB/c mouse serum collected 50 days following inoculation with 1 x 10^6 LV39 *Lm* promastigotes and subsequent treatment with PBS (Control), AmBisome (3 mg/kg or 5mg/kg AMB) or AMB-ND (3 mg/kg or 5 mg/kg AMB) at 24 h, 48 h, 10 days, 20 days, 30 days, and 40 days. Images collected with Adobe Photoshop 6.0 & Windows Powerpoint 2003.

Table 3.1. Effect of AMB-ND therapy on cytokine production in *L. major* infected mice. BALB/c mice were infected with 1 x 10^6 LV39 *L. major* promastigotes followed by treatment with PBS or AMB-ND (1 and 5 mg/kg AMB) at 24 h, 48 h, 7 days, 14 days, and 21 days. Lymph node culture supernatants were obtained at specified time points following infection and assayed for cytokine production. Values reported are the mean ± standard deviation of ELISA measurements (n = 3) from 2 separate experiments.

	Untreated Controls	1 mg/kg AMB-ND	5 mg/kg AMB-ND
IFN-γ (ng/mL)			
Day 15	85.12 ± 51.29	31.99 ± 13.81	42.32 ± 48.01
Day 30	12.75 ± 7.51	14.51 ± 14.65	2.08 ± 2.03
Day 50	15.29 ± 7.14	26.72 ± 5.46	31.66 ± 10.24
Day 140		14.40 ± 2.34	38.97 ± 0.28
Day 250			114.7 ± 3.6
IL-12 (ng/mL)			
Day 15	173.33 ± 78.76	41.96 ± 49.75	138.30 ± 18.38
Day 30	0	13.86 ± 27.73	88.72 ± 115.25
Day 50	0	7.50 ± 15.00	0
Day 140		0	0
Day 250			66 ± 3.3
NO (μM/mL)			
Day 15	2.40 ± 0.38	0.44 ± 0.45	0.39 ± 0.46
Day 30	0.03 ± 0.06	0.71 ± 0.83	0.094 ± 0.19
Day 50	2.31 ± 2.23	0.71 ± 0.64	0.54 ± 0.29
Day 140		4.69 ± 4.15	1.29 ± 0.39
Day 250			0.4 ± 0.01
IL-4 (pg/mL)			
Day 15	136.82 ± 84.70	109.87 ± 51.96	54.43 ± 12.29
Day 30	104.55 ± 18.68	80.06 ± 10.41	75.00 ± 51.43
Day 50	95.44 ± 32.63	75.03 ± 9.76	121.45 ± 55.53
Day 140		73.03 ± 4.57	10.43 ± 2.05
Day 250			11.50 ± 0.64
IL-10 (ng/mL)			
Day 15	166.60 ± 29.96	129.23 ± 51.86	72.20 ± 58.77
Day 30	27.97 ± 32.32	33.72 ± 36.03	45.94 ± 52.12
Day 50	96.05 ± 37.23	57.31 ± 18.67	70.93 ± 21.67
Day 140		28.79 ± 8.66	25.05 ± 2.43
Day 250			28.30 ± 5.20

Table 3.2. Effect of AMB formulation on cytokine production in *L. major* infected mice. BALB/c were infected with 1 x 10^6 LV39 *L. major* promastigotes and treated with PBS, 5mg/kg AMB in AmBisome, or 5 mg/kg AMB in AMB-ND at 24 h, 48 h, 10 days, 20 days, 30 days, and 40 days. Lymph node culture supernatants were obtained at specified time points and analyzed for cytokine production. Values reported are the mean ± standard deviation of ELISA measurements (n = 3) from one experiment.

	Untreated Controls	5 mg/kg AmBisome	5 mg/kg AMB-ND
IFN-γ (ng/mL)			
Day 14	24.6 ± 2.9	18.6 ± 0.92	23.4 ± 0.76
Day 36	0.06 ± 0.002	0.62 ± 0.02	1.2 ± 0.05
Day 50	3 ± 0.23	3.4 ± 0.24	0.58 ± 0.08
Day 95		1.6 ± 0.36	1.64 ± 0.35
Day 140			39.3 ± 7.5
IL-12 (ng/mL)			
Day 14	68 ± 8.7	109 ± 7.1	134 ± 13
Day 36	18.9 ± 7.2	33.6 ± 7.9	14.5 ± 9
Day 50	64.2 ± 5.6	44.7 ± 26.8	56.9 ± 16.2
Day 95		49.4 ± 3.1	38.8 ± 6
Day 140			17.3 ± 4.6
NO (μM/mL)			
Day 14	0.17 ± 0.02	0.03 ± 0.01	0.11 ± 0.02
Day 36	0	0.2 ± 0.07	0.03 ± 0.01
Day 50	0.28 ± 0.02	0.34 ± 0.03	0.2 ± 0.01
Day 95		0.08 ± 0.01	0.05 ± 0.01
Day 140			0
IL-4 (pg/mL)			
Day 14	11.7 ± 0.87	10.5 ± 1.08	3.6 ± 0.61
Day 36	5.2 ± 0.55	8.4 ± 0.9	4.5 ± 0.53
Day 50	6.2 ± 0.43	6.8 ± 0.35	16.4 ± 3.1
Day 95		4.6 ± 0.26	2.8 ± 0.15
Day 140			42.7 ± 2.8
IL-10 (ng/mL)			
Day 14	160 ± 14	137 ± 17	160 ± 31
Day 36	49.2 ± 4.3	78.8 ± 4.4	51.6 ± 1.7
Day 50	69.3 ± 3.8	71.2 ± 7.4	91 ± 6.6
Day 95		53.5 ± 4.3	47.3 ± 3.6
Day 140			90.6 ± 1.2

DISCUSSION

In this study, we evaluated a novel lipid formulation of AMB in treatment of *L. major* infection in susceptible BALB/c mice. The data reveal that non-lethal doses of AMB-ND eradicate an experimental cutaneous *L. major* infection. Numerous studies have confirmed the efficacy of AMB lipid formulations in treatment of visceral *Leishmania* infection, achieving clearance rates of up to 96% of organisms with high dose treatment and long-term recrudescence rates as low as 10-20% (Murray et al., 2000a; Escobar et al., 2001; Ehrenfreund-Kleinman et al., 2005). Similar studies have shown increased efficacy and decreased toxicity of liposome-complexed AMB in treatment of experimental *L. infantum* infection (Gangneux et al., 1996). Treatment of cutaneous *L. major* infection with AMB lipid formulations (specifically AmBisome) has resulted in temporary reductions in lesion size without complete clearance of the parasites (Yardley and Croft, 1997; Yardley and Croft, 2000). To assess the efficacy of AMB-ND, we compared IP-injected AMB-ND, empty ND and AmBisome with respect to their ability to decrease pathology (e.g. cutaneous lesion size) and parasite burden in *L. major* infected BALB/c mice. The results show that, at 5 mg/kg AMB, under the treatment regimen employed, AMB-ND elicit a marked improvement over AmBisome in terms of pathology, parasite burden and survival. At the same time, empty ND were ineffective. AmBisome has been reported to be effective for temporary lesion resolution at an intravenous (IV) 25-50 mg/kg dose for a 6-12 day, 6 dose treatment regimen, with lesions dramatically reduced over the following 4 weeks, although lesions recurred within 7-8 weeks post-treatment (Yardley and Croft, 1997; Yardley and Croft, 2000). Based on the fact that parasite inoculum, treatment frequency and dosage of AMB were identical in the

ND and AmBisome treatment groups, the data suggest that some aspect of the ND particle structure may be enhancing the therapeutic potential of the antibiotic. A potential confounding factor may be the administration of AmBisome and AMB-ND via the IP route, rather than IV, as it is known that AmBisome has reduced effectivity when administered subcutaneously near lesions and may be less effective when administered IP (Yardley and Croft, 1997). However, the same might well be true for AMB-ND, and future studies using IV administration of AMB-ND will address this question. Mechanisms for increasing the effectiveness of the AMB-ND formulation may include increased time in circulation, bioavailability or increased delivery of AMB-ND complexes to the infected macrophages.

Lipid complexes have been used for many modalities of drug delivery and have proven to be efficacious in both reducing toxicity and increasing the circulation residence time of associated drugs [Basu and Lila, 2004; Medda et al., 2003 (liposomes, phospholipid microspheres); Gangneux et al., 1996 (AmBisome); Yardley and Croft, 2000 (Ambisome, Amphocil, Abelcet)]. Drug clearance time for AmBisome, in particular, can be dramatically decreased in various tissue compartments, as compared to the blood (Gangneux et al., 1996). ND are lipid complexes and, as such, they may have an altered clearance time, potentially producing enhanced effects at lower dosage frequency. Experiments to assess this were not specifically performed but circumstantial evidence, consisting of the observed long-term effects of AMB-ND treatment on parasite numbers, wherein parasite burden continued to decline for 100+ days following the final AMB-ND treatment at day 21 or 40 post-infection (see **Figures 3.2 and 3.4**), suggest the ND lipid milieu may protect associated AMB from degradation or clearance for weeks to

months following administration, thus producing the long-term post-treatment effects seen.

Another possibility is that ND are recognized by the class A scavenger receptor (SR-A) on macrophages, the tissue site of *L. major* replication. It is known that apolipoproteins, modified by acetylation, oxidization or other modifications serve as ligands for SR-A. Although the apolipoprotein component of AMB-ND (recombinant human apolipoprotein A-I) has not been deliberately modified, recognition of non-self lipoproteins by the murine SR-A cannot be excluded at present. It has been shown that activated macrophages increase expression of scavenger receptors on their surfaces (Tempone et al., 2004) and macrophages infected with *L. chagasi* have increased scavenger receptors on their surface (Broz et al., 2005), suggesting that *Leishmania* phagocytosis results in up-regulation of SR-A. Others have observed co-localization of phagolysosome compartments and scavenger receptor-targeted molecules (Tempone et al., 2005), demonstrating delivery of SR-A ligands to the phagolysosome. Increased delivery of AMB-ND to the parasite-containing macrophage population or direct targeting to the phagolysosome compartment would be expected to result in increased efficacy of AMB-ND in *Leishmania* infected mice. This hypothesis needs further investigation, including assessment of the interaction between macrophage scavenger receptors and ND, as well as investigation of co-localization of ND and phagolysosomes.

With many of the experimental and clinical anti-cutaneous leishmaniasis therapeutics, a degree of immune response is necessary, as in the case of the pentavalent antimonials (Nabors et al., 1995; Li et al., 1997; Murray et al., 2000b), where Th2 to Th1 switching via IL-12 or IFN-γ is necessary for complete clearance. For this reason, we

assessed several Th1 (IL-12, IFN-γ, NO) and Th2 (IL-4, IL-10) cytokines to investigate the possible role of the cytokine immune response in AMB-ND treatment of cutaneous leishmaniasis. In the present study, we observed no significant differences in any of the cytokines assayed between AMB-ND treatment groups and those treated with PBS or empty AMB-ND alone. These data indicate there is no requirement for a change in the cytokine response for full and lasting clearance of *L. major* from BALB/c mice with AMB-ND treatment. Furthermore, these results suggest AMB-ND treatment may function equally well in immuno-compromised patients, thereby increasing its potential utility in HIV-leishmaniasis co-infection therapy. Although some evidence exists that AMB clearance of *Leishmania* infection is non-immune status dependent, no studies to date have shown definitive clearance of cutaneous *Leishmania* infection without immunotherapy intervention.

As in the control treatment group, mice treated with AMB-ND developed antibodies to *L. major* (see **Figure 3.5**). Differences between the antigen banding patterns in the 5 mg/kg AMB-ND group versus all other groups, however, suggests there is some fundamental difference in antibody production associated with early clearance and failure to establish a full *L. major* infection in this treatment group. Decreased antibody binding suggests that antibody production was reduced in this group. A similar decrease in antibody reactivity has been reported in humans and dogs with spontaneous or chemotherapeutic-associated healing of *Leishmania* spp. infections (Brito et al., 2001; Moreno et al., 1999). The decreased antibody reactivity in the 5 mg/kg AMB-ND treatment group is possibly associated with early clearance of the majority of inoculated *L. major*, leaving fewer parasites to produce antigens and thus reduced antigen exposure

for the host over the course of the infection, as well as parasite destruction leading to a different antigen profile, including cytoskeletal, organelle, and other antigens from dead or dying *L. major*.

In conclusion, AMB-ND represent a remarkably effective therapy for experimental cutaneous *L. major* infection in BALB/c mice. At equivalent dosage and treatment frequency it far surpasses commercially available liposomal AMB and results in sterile clearance of *L. major* infection using a limited and widely spaced therapeutic regimen. The absence of statistically discernable changes in the cytokine response indicates that this therapy is not dependent on host cytokine-based immunity for sterile clearance. Future investigations will explore the interaction between ND and macrophage scavenger receptors. In addition, investigations of the tissue and the plasma pharmacokinetics of ND-associated AMB and toxicity studies of organs affected by AMB, specifically including the renal and hepatic systems, are planned. Given the versatility of the ND formulation with respect to its intrinsic protein component, protein engineering may be used to specifically target ND to macrophages.

CONCLUSION

Interaction between the vertebrate host and *Leishmania major* may influence the progression of disease and long-term response to the parasite by the host. Interactions between complement, particularly complement component 3 (C3), and parasite surface molecules LPG and gp63 are of particular interest, as opsonization of parasites may affect recognition, receptor activation, and subsequent immune response by phagocytic cells and downstream immune cells. We utilized C3 knockout (C3KO) mice on a *L. major*-resistant background and LPG-deficient, gp63-deficient, and wild type LV39 *L. major* parasites to investigate this interaction.

The eventual development and clearance of leishmanial lesions *in vivo* suggests that C3 and opsonization are not essential to *L. major* phagocytosis and infection, but that they alter the host response to the parasite. If C3 was essential for phagocytosis and progression of *L. major* infection, then infection of C3KO mice with *L. major* would result in no disease progression and certainly no increases in parasite numbers over time. However, it is obvious that C3 plays a role in normal *L. major* infection, as C3KO animals have noticeable changes in the progression of not only lesions, but also parasite amplification and cytokine responses to *L. major*. The role of C3 may be one of opsonization, allowing increased and more efficient phagocytosis of *L. major*, but without being the sole ligand for stimulation of phagocytosis of *L. major*. Thus the initially reduced parasite burden and lesions, which would indicate that the histiocytic response was reduced and that *L. major* phagocytosis (and therefore protection) was

reduced in early infection. Presumably this was due to the lack of C3 opsonization and resultant enhancement of phagocytosis. However, as there are other routes by which *L. major* may bind to the phagocytic cells and induce phagocytosis, there is an eventual increase in parasite burden and lesion size above that of the controls.

Along with this is the observation that C3 must be involved not only in phagocytosis of *L. major*, but also in determining survival and long-term immune response to the parasite. This is born out by the increased length of infection seen in C3KO animals, suggesting that lack of C3 results in not only inefficient phagocytosis, but also inefficient clearance of parasites. This increased clearance time for *L. major* infection suggests that C3 opsonization and the development of a Th1 response are linked to some degree, with C3 opsonization of *L. major* allowing normal development of a Th1 response with rapid parasite clearance. Similar conclusions may be drawn from the cytokine responses of C3KO mice, which lag behind those of resistant mice, suggesting that there is a decreased ability of the immune system to initially recognize and respond to the parasite. However, cytokine responses remain Th1 in nature and do not switch to Th2-type responses, suggesting that C3 is not necessary for development of the Th1 response in otherwise resistant mice.

The *in vivo* results suggest that LPG is extremely important in both host response and parasite survival *in vivo*, such that parasites without LPG are essentially ineffective at infection of the host or production of a host response. The role of gp63 in parasite recognition and host response to *L. major* is apparently interlinked with the role of complement as an opsonin for macrophages. This is seen in the *in vivo* response to gp63KO parasites, in which the time course of lesion development and parasite growth

was altered in resistant control animals to resemble that seen in C3KO mice. It is obviously not necessary for infection, as is LPG, but is nevertheless an important part of host recognition and infection.

In vitro, as *in vivo*, the role of LPG is a vital one, with greatly decreased infection of naïve macrophages by LPGKO parasites, as well as decreased levels of cytokine and monokine production compared to wild-type parasite infections. The relatively equal levels of naïve macrophage phagocytosis despite blocking of different complement receptors suggests that LPG is a key component in parasite interactions with multiple macrophage surface receptors and a deficiency in LPG has wide-ranging effects that are not limited to just certain complement receptors. This is similar to the broad-based changes in infection profiles seen in the *in vivo* experiments. The variance in cytokine and monokine levels seen with blocking of specific complement receptors suggests that there is recognition by specific complement receptors of different aspects of the parasite, and LPG is not the sole ligand for either phagocytic recognition/stimulation or cytokine/monokine production by any one given receptor.

In vitro, gp63 seems to be involved to some degree in interactions between both CR1/CR2 and CR3, although CR3 interactions are more severely affected, both in terms of phagocytosis and cytokine and/or monokine production. Again, this reinforces the *in vivo* data, which suggests that gp63KO-infected resistant mice can have temporal changes in the infection profile similar to that seen in C3KO mice infected with wild-type *L. major*. As the C3 product, iC3b, is one of the major ligands for CR3 in *L. major* infection, this would suggest that the interaction between gp63 and C3 seen *in vivo* is reflected in the *in vitro* data involving gp63 and CR3. Neither LPG nor gp63 appear to

be significantly responsible for the development of a Th1 vs. Th2 response bias, either alone or in interaction with CR1/CR2 and/or CR3.

The *in vitro* results also suggest that there is no one specific mechanism by which the *L. major* parasite is recognized, stimulates a cytokine/monokine response, or is phagocytosed. Complement receptor interactions are obviously complex and involve multiple redundant pathways of macrophage recognition and response to the parasite. Regulation and limiting the complement receptors exposed *in vitro* has no observable effect on the Th1 vs. Th2 bias seen in the cytokine response, even though there are differences in the degree of cytokine response produced by varying the complement receptor blocking. These cytokine and phagocytic differences may result in some of the *in vivo* responses seen, but likely only in combination with a host of other factors that are less easily defined.

The temporal lag observed *in vivo* in the response of the C3 KO mice to *L. major* infection and the variance in phagocytosis when CR3 and/or CR1/CR2 are blocked *in vitro* indicates that C3 opsonization is one of the more significant routes by which the parasite is recognized and phagocytosed by macrophages and possibly one of the mechanisms that induces the host's innate/adaptive immune response. However, since there is still a response to the parasite in the C3KO mice, opsonization is not required for parasite recognition and host response. Since C3 opsonization is not required, other ligands and receptors therefore must be utilized by host macrophages in recognition and phagocytosis of *L. major*. Among possible receptors may be macrophage scavenger receptors, mannose receptors, and Toll-like receptors, which may recognize ligands other than C3b and iC3b, including *L. major* LPG and/or gp63.

The success of macrophage scavenger receptor-targeted chemotherapeutics in *L. major* treatment suggests that macrophage receptor-mediated or targeted therapies may be of significant importance in treatment of leishmaniasis and even protection against leishmaniasis. Additionally, this mode of therapeutic targeting of anti-leishmanial drugs may also be of use in targeting treatments for other intra-histiocytic organisms, including fungi, bacteria, and protozoa. Certainly, further exploration of broad-based targeting of macrophage receptors involved in recognition of and response to *Leishmania*, including scavenger receptors and complement receptors would be of great utility in devising anti-leishmanial therapies.

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