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Interleukin-3 promotes susceptibility in a mouse model of Cutaneous Leishmaniasis

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Dedication

This thesis is dedicated to my mother and father for their continued support through all my endeavors. I especially want to thank them for always expressing interest in my research and believing in my capabilities. Also, I thank Grandpa LeRoy, Grandma Becky, and Grandpa and Grandma Sayre for always pushing me to become a better person and convincing me to pursue my dreams.

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Abstract

Cutaneous leishmaniasis (CL), a vector-borne infectious disease caused by protozoan parasites of the genus *Leishmania*, is one of the most important neglected infectious diseases worldwide. Currently, 10 million people in 82 (mostly developing) countries are infected. Those infected develop ulcerative skin lesions on exposed parts of the body, causing serious disability and permanent scarring. All mice are susceptible to infection with *Leishmania major*, however, the outcome of infection is different depending on the mouse strain. For example, resistant C57BL/6 mice develop lesions, which like in humans, ultimately heal. In contrast, lesions in susceptible BALB/c mice progressively worsen, ultimately resulting in mortality. Resistance or susceptibility to *Leishmania* parasites is largely dependent on whether the host's CD4$^+$ T cells develop into Th1 or Th2 effector cells, respectively. Resistant Th1 responses are typically driven by cytokines like interleukin (IL)-12 and interferon-γ which promotes healing and parasite clearance. In contrast, susceptible Th2 responses are marked by elevated IL-4 production which inactivates the mechanisms of effective parasite clearance and promotes disease progression through an ineffective, antibody-dominated humoral response. IL-3 is a cytokine which promotes hematopoiesis and has the ability to act on numerous cell lineages. In particular, IL-3 appears to have specialized functions in regards to the activation of basophils. For example, IL-3 has been shown to be indispensable for increases in basophil numbers in response to certain infections. Moreover, IL-3 has been shown to increase the functional ability of basophils as IL-3-stimulated basophils are shown to secrete higher levels of IL-4. IL-3 is primarily secreted by activated CD4$^+$ effector T cells, the same cells which are so influential in determining resistance and susceptibility to CL. However, the role of IL-3 during the response to CL remains largely unknown. In the present study we show that

infected mice genetically deficient in IL-3 (IL-3 -/- mice) develop smaller lesions, have a lower parasite burdens, and express lower levels of B cells in draining lymph nodes as compared to infected IL-3 +/+ mice. These data suggest IL-3 promotes susceptibility to *Leishmania* infection and may play an important role in the development of a Th2 immune response characteristic of susceptible BALB/c mice.

Introduction

Cutaneous Leishmaniasis

Cutaneous Leishmaniasis (CL) is a vector-borne disease induced by multiple protozoan species of the genus *Leishmania*. Transmission of this disease to a susceptible individual leads to formation of ulcerated lesions in the dermis. Although lesions ultimately heal after a few months, they often lead to secondary bacterial infections and can leave permanent scarring and disfigurations. CL is a major threat to developing countries, although cases are reported virtually worldwide. Overall, incidents of CL are concentrated in certain areas with over 90% of all cases being reported in Afghanistan, Algeria, Iran, Iraq, Saudi Arabia, Syria, Brazil, and Peru (1). The United States Department of Defense has recently heightened their interest in this disease due to the deployment of U.S. troops into regions where leishmaniasis is endemic (2). Exposure to *Leishmania* parasites in these regions has caused the number of cases in the U.S. to increase to a level not seen since World War II (3). Currently, the Centers for Disease Control and Prevention (CDC) reports *Leishmania* infection has become endemic to 88 countries and causes 1.5 million new cases of CL each year. Although these numbers are staggering, a true approximation of how many people this disease affects is impossible to assess due to misdiagnosis, unreported cases, and insufficient communication with health officials.

Leishmania major (L. major) is the causative agent of zoonotic CL. This protozoan parasite has a digenetic life cycle made up of two developmental stages (4). The first is an extracellular developmental stage located inside the sand fly vector where the parasite assumes a spindle, flagellated morphology called a promastigote (Fig. 1). The second developmental stage is intracellular and occurs primarily within host mammalian

Figure 1 Microscopic image of flagellated *L. major* promastigotes in culture (1000 x magnification).
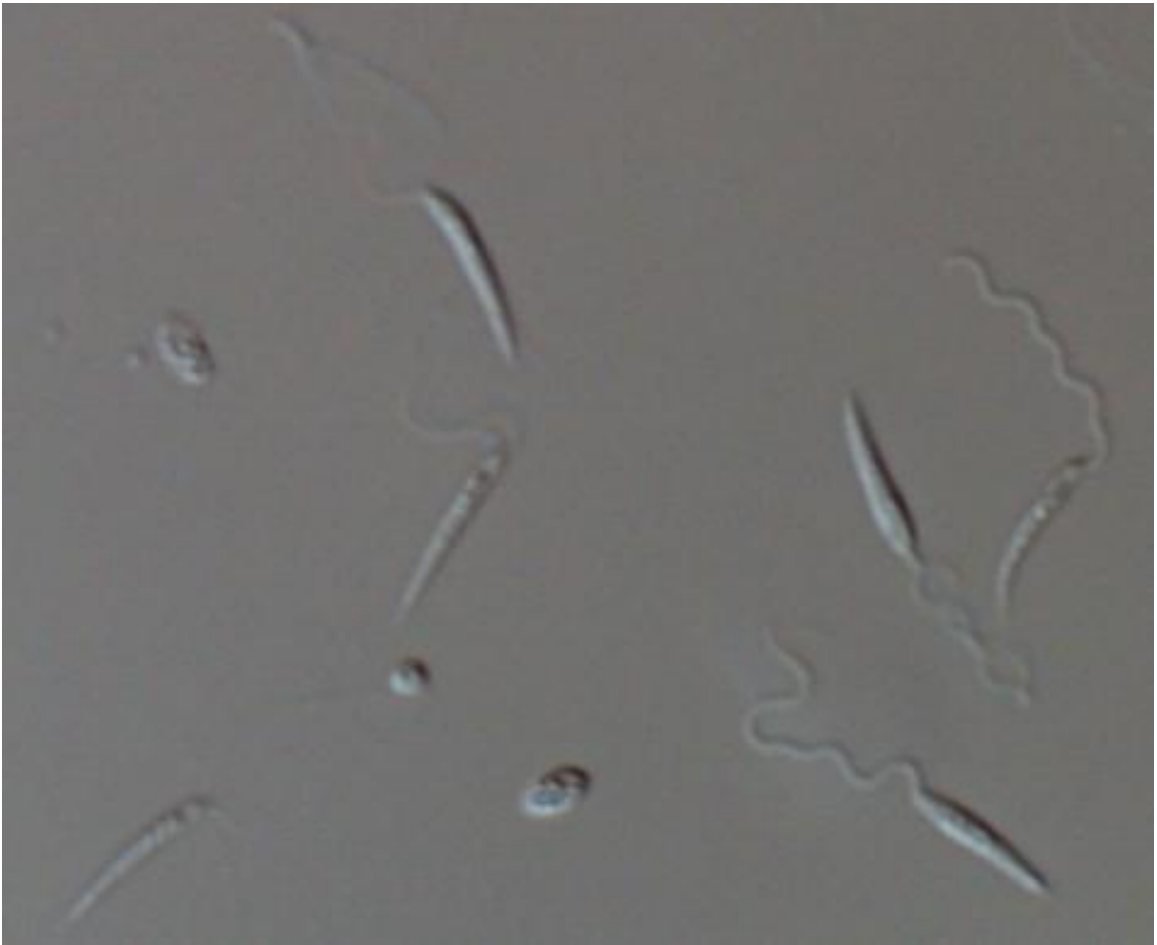
macrophages where parasites take on a smaller, non-flagellated ovoid shape referred to as an amastigote (4, 6). The complete life cycle of *Leishmania* parasites is depicted in Figure 2.

The first stage of the parasite's life cycle begins when the sand fly ingests macrophages containing *Leishmania* amastigotes while feeding on a mammalian host. Once ingested, infected macrophages travel to the midgut of the vector where they lyse, releasing the amastigotes. Upon release, amastigotes transform to immature promastigotes termed procyclics, whose surface is coated with lipophosphoglycan (LPG) (7). On immature *Leishmania* promastigotes LPG molecules bind to insect gut epithelial cells (7) to provide stability and allow for parasite maturation.

Leishmania parasites undergo development in the sand fly referred to as metacyclogenesis. This maturation results in modifications to the LPG molecules causing them to elongate and dissociate from gut epithelial cells rendering the parasites motile (7). Metacyclogenesis produces mature parasites called metacyclic promastigotes, which are infective to humans and other mammalian hosts (4). Infected sand flies inject these infective parasites into a new host while taking a blood meal, thus beginning the second developmental stage in mammals.

Metacyclic promastigotes present within the mammalian hosts, quickly become engulfed by macrophages (8). Parasites lose their flagellum inside these cells and transform to amastigotes, the developmental stage in which they remain in the mammalian host (7). Macrophages provide a venue for parasitic division which is accomplished by binary fission (7). Replication ultimately lyses the host cell releasing amastigotes, which can infect other phagocytes. Parasites are transmitted back to the sand fly when cells harboring amastigotes are ingested from infected hosts during subsequent blood meals.

Innate immune response to L. major

Like other pathogens, *L. major* parasites are quickly confronted by effector

Figure 2 Life cycle of *Leishmania* spp. showing developmental stages in both the mammalian host and sand fly vector (8).
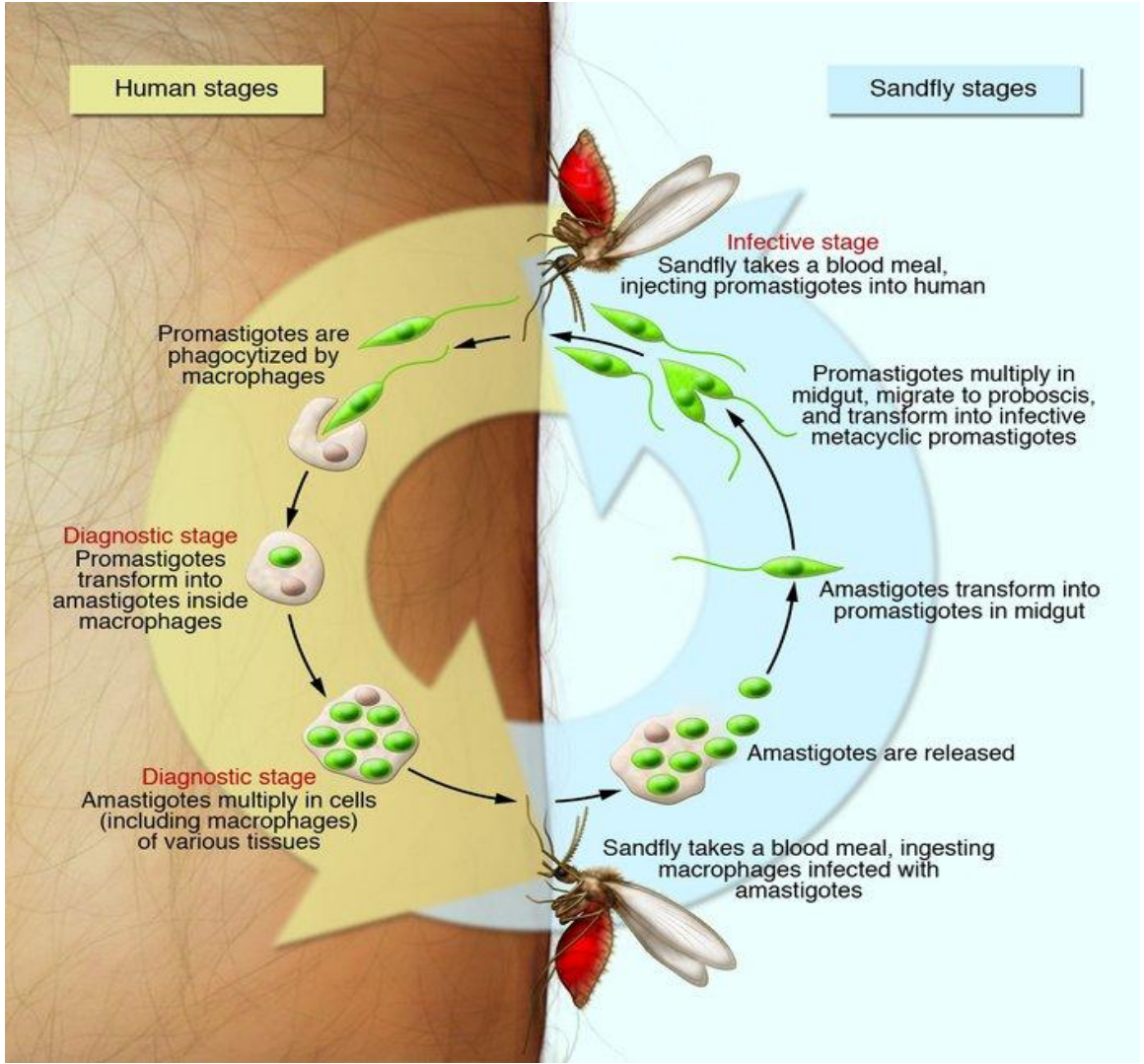
mechanisms of the innate immune system such as complement proteins. The complement system is comprised of numerous proteins that function to work against pathogens immediately following infection. Normally, there are two primary outcomes for pathogens which activate the complement system. First, complement can form a membrane attack complex (MAC) which perforates the membranes of invading pathogens causing them to degrade. Alternately, complement can opsonize pathogens allowing phagocytes expressing complement receptors to bind and engulf these pathogens.

L. major metacyclic promastigotes, the infective stage for the mammalian host, are able to avoid the detrimental effects of MAC formation by the alterations which occur during metacyclogensis. In both non-infective promastigotes and metacyclic promastigotes, LPG molecules are the primary binding site for complement proteins (9). However, as metacyclic promastigote LPG molecules become elongated, they are able to prevent the complete formation of MACs. This was demonstrated by Puentes *et al.* who noted effective MACs formed on LPG molecules of non-infective promastigotes, whereas MACs could not form on LPG molecules of metacyclic promastigotes (10). This finding suggests the longer LPG molecules seen on metacyclic promastigotes may act as a physical barrier, inhibiting the formation of intact MACs.

Another way *L. major* is able to avoid initial immune responses is through their ability to hide within cells such as macrophages, which are typically responsible for eliminating pathogens. Macrophage uptake of parasites is thought to occur in a number of different ways. For example, Russell *et al.* (1988) demonstrated phagocytosis of *Leishmania* parasites occurred through binding of complement receptor CR3 on macrophages directly to gp63, a major surface glycoprotein expressed on promastigotes (11). Additionally, macrophage uptake of *Leishmania* promastigotes has been shown to occur indirectly by complement

receptors CR1 and CR3 binding C3b and iC3b opsonized LPG molecules, respectively (9, 12). Collectively, these studies suggest macrophage internalization of *Leishmania* promastigotes can happen in a number of different ways and could be dependent upon complement proteins. Once located intracellularly, *L. major* must use a number of specializations to survive in the hostile environment of macrophages.

When promastigotes are engulfed, they become surrounded by the plasma membrane of macrophages forming a compartment called a phagosome. Lysosomes inside the host macrophage then fuse with phagosomes to form parasitophorous vacuoles (PVs), the site where promastigotes develop into amastigotes (13). Survival at this stage requires that parasites be able to withstand the harsh, protease rich environments of PVs that can reach pH values as low as 4.74 (14). It has been shown that parasites express a variety of molecules which aid their survival in this environment (15, 16). However, the adjustments made by amastigotes to avoid destruction in PVs are not fully understood and will require further investigation. Eliciting these mechanisms may reveal important targets for therapeutics in the fight against CL.

Engulfment of *Leishmania* parasites induces cytokine and chemokine production by cells at the site of infection. Released cytokines and chemokines recruit other immune cells to the site of infection. As immune cells begin trafficking to the infection site, antigen presenting cells (APCs), such as skin dendritic cells (a.k.a. Langerhans cells), become infected and then proceed to the draining lymph nodes (LNs) of the host (17). Dendritic cells then initiate adaptive immune responses by presenting the parasites to T helper (Th) lymphocytes expressing T cell receptors (TCRs) specific for *Leishmania* antigens. These TCRs were found to primarily recognize a particular *Leishmania*-peptide sequence which was designated LACK (*Leishmania* homolog of the receptor for activated C Kinase) (18). Once *Leishmania*-specific

Th lymphocytes become primed, they differentiate into subpopulations of effector cells which initiate adaptive immune responses against the parasites.

T helper 1/T helper 2 cell paradigm

A pivotal point in the immune response is when CD4$^+$ T helper cells differentiate into sub-populations such as T helper 1 (Th1) and T helper 2 (Th2) (Fig. 3). Naive CD4$^+$ T cells become activated in lymphoid tissue by APCs expressing antigens in the context of a molecule known as the Major Histocompatibility Complex (MHC) Class II. APCs such as dendritic cells, located at the initial site of infection, take up pathogens and process their antigens by the endocytic pathway. This ultimately results in the expression of the pathogen's antigenic peptides on the surface of APCs. During antigen processing, APCs make their way to secondary lymphoid tissues such as the draining LNs and spleen where they present pathogen derived antigenic peptides to naive CD4$^+$ T cells. If CD4$^+$ cells have TCRs specific for the particular antigen being presented, they will undergo clonal expansion and differentiation into effector cell subsets such as Th1 and Th2 cells. Th1 and Th2 cell differentiation is regulated by cytokines and each subset of cells has distinct effector mechanisms.

Whether a naive T helper cell becomes a Th1 or Th2 cell primarily depends on the type of cytokines present in the cellular environment during the early stages of infection. If secondary lymphoid tissue is rich in cytokines like interleukin-12 (IL-12) and interferon-γ (IFN-γ), T cells will predominately develop into Th1 cells (19). After differentiation, CD4$^+$ Th1 cells can exit lymphoid tissue and activate CD8$^+$ T cells, macrophages, and other phagocytic cells which are effective at eliminating intracellular invaders (20-22). For example, Th1 cells are able to activate macrophages causing them to boost production of nitric oxide

Figure 3 Schematic representation of naive CD4$^+$ T helper cells differentiating into subpopulations in response to different cytokines (Figure modified from Mesquita *etal*. (23)).

NAIVE CD4$^+$ T CELL

IFN-γ	IL-4
Th1	Th2
IFN-γ, IL-2	IL-4, IL-5, IL-13
-Response to intracellular pathogens -Activation of phagocytes -Production of opsonizant antibodies -Delayed hypersensitivity	-Response to extracellular pathogens -B cell proliferation -B cell differentiation -Antibody production -Eosinophil activation
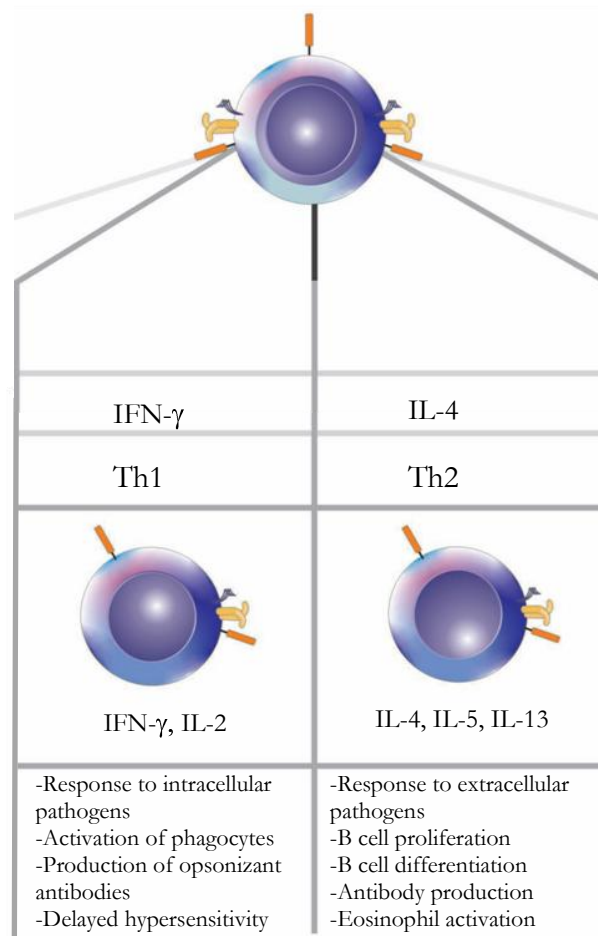
and its derivatives which results in a hostile intracellular environment for pathogens (23).

In contrast, naive CD4$^+$ T cells in the presence of cytokines such as IL-4 and IL-10 develop into Th2 cells (19). CD4$^+$ Th2 cells primarily function to stimulate activated B cells to further differentiate into antibody (Ab) secreting plasma cells, thus leading to a humoral-dominated immune response. Th2 polarized immunity has been shown to effectively combat extra cellular pathogens such as helminths (24) and is associated with promoting the pathology seen in asthma and other IgE-dependent allergic diseases (25).

Th1 and Th2 lymphocytes in murine CL

Th2 immunity produces responses which are primarily effective against extracellular pathogens while Th1 immunity is better equipped for eliminating intracellular pathogens. Because *Leishmania* parasites remain intracellular within the mammalian host, to clear this infection a host must mount an INF-γ– and IL-12-driven Th1 response. There are well-established mouse models used to study host immune responses against CL. The most popular of these models utilizes genetically susceptible BALB/c mice and resistant C57BL/6 mice (26).

In susceptible strains such as BALB/c, *Leishmania* infection induces Th2 type immunity resulting in elevated Ab levels, high IL-4, and low IFN-γ expression (21) resulting in progressive disease and parasite visceralization which ultimately becomes lethal to BALB/c mice (27). In contrast, resistant C57BL/6 mice mount a Th1 polarized immune response to *Leishmania* infection that is characterized by modest Ab levels, high IFN-γ, and low IL-4 expression (27). This type of immunity upregulates the production of reactive oxygen species by *Leishmania*-infected macrophages, which impedes parasite growth and results in disease clearance (28).

Direct evidence for the necessity of a Th1 response in clearing *Leishamania* infection has been shown by using purified Th1 and Th2 cells specific for *Leishmania* antigen (Ag) and transferring them into susceptible BALB/c mice prior to infection (29). These studies were able to show that *L. major*-infected mice which were passively immunized with *L. major*-specific Th2 cells displayed accelerated lesion development compared to control mice. However, susceptible BALB/c mice were protected from infection following passive immunization with *L. major*-specific Th1 cells.

Interleukin-3

Interleukin-3 (IL-3) is a cytokine primarily secreted by CD4$^+$ T cells which have become activated by antigens during infection (30). This cytokine is a 28-kDa glycoprotein comprised of 133 amino acids and has been implicated in the expansion of hematopoietic progenitor cells (31). This expansion leads to increased production of cells such as neutrophils, macrophages, eosinophils, erythrocytes, megakaryocytes, dendritic cells, mast cells and basophils (31). In addition to expanding the population of its target cells, IL-3 also appears capable of increasing the functional ability of these cells, which affects the levels of secretory product expressed by target cells.

Once secreted, IL-3 binds to target cells expressing IL-3 receptors composed of two subunits. The first receptor component is an alpha subunit (IL-3Rα) which is specific for IL-3. The second receptor component is referred to as the beta common (βc) subunit, which is shared by the receptors for IL-3, IL-5, and granulocyte macrophage-colony stimulating gactor (GM-CSF) (32). Both the α and βc subunits are required for IL-3 to optimally activate target cells (33). However, it is important to note that IL-3 can utilize an additional βc subunit called βc^{IL-3}, which is not shared among IL-5 and GM-CSF receptors (34).

Therefore, in mice which are lacking a functional βc subunit, IL-3 can remain functional by binding IL-3Rα and $βc^{IL-3}$ subunits.

The process of IL-3 activation begins when the cytokine associates with an IL-3-specific α subunit. This interaction produces a low affinity binding complex which is not sufficient for activating the signaling pathways which stimulate target cells. However, in the presence of the βc or $βc^{IL-3}$ subunits, the affinity of IL-3 for the receptor increases causing the receptors to aggregate and transmit signals to internal regions of cells (35). Aggregated βc (or $βc^{IL-3}$) subunits allow enzymes located in the cytoplasm of target cells to phosphorylate these receptors, a mechanism required for IL-3 signaling (36).

Once neighboring βc subunits aggregate, they interact with tyrosine kinases known as Janus kinases (JAKs). Although there are a number of JAKs proteins which have been identified, JAK-2 appears to dominate in the activation of IL-3 receptors (37). Activity from JAK-2 results in the phosphorylation of multiple tyrosine residues, which converts them to binding sites for intracellular signaling proteins (38, 39). The most important intracellular signaling proteins for IL-3 activation of target cells are proteins called signal transducers and activators of transcription (STATs) (40). STATs dock to the phosphorylated tyrosine residues and transmit signals to the cell's nucleus activating transcription of particular genes (33). Activating transcription pathways results in the stimulation of target cells and leads to proliferation and increased cellular function.

IL-3 and Leishmania infection

There have only been a limited number of studies suggesting that IL-3 functions during immune responses to *Leishmania*. Lelchuk *et al.* (1988) suggested a role for IL-3 in exacerbating CL following experiments which used spleen cells from genetically resistant and

susceptible mice strains (41). These researchers showed cells isolated from resistant C57BL/6 mice generated lower levels of IL-3 when exposed to *Leishmania* antigens compared to cells isolated from susceptible BALB/c mice. These studies suggest the presence of IL-3 may be at least partially responsible for susceptible mice strains mounting an ineffective immune response to *Leishmania* parasites.

Other investigators evaluating IL-3 in CL include Feng *et al.* (1988) who experimentally infected footpads of susceptible BALB/c mice with *L. major* (42). After infection, investigators injected recombinant IL-3 into the mice and then assessed lesion sizes. They found that infected mice that were treated with IL-3 had larger lesions compared to mice treated with Phosphate Buffered Saline (PBS) as a control. These findings were supported by Saha *et al.* (1999) who also experimented with *L. major* infected mice (43). These investigators injected mice with *Leishmania* parasites and then treated them with neutralizing antibodies against murine IL-3. They found disease severity was markedly reduced in mice given anti-IL-3 antibodies, which in accordance with the above studies, indicated IL-3 promotes susceptibility to *Leishmania* infection in BALB/c mice. Collectively, the results from these experiments suggest susceptibility to CL is influenced by IL-3.

Basophils

IL-3 production can influence the number and activational status of most hematopoietic cells, especially basophils. Basophils are granulocytes that compose a very small portion of peripheral white blood cell (WBC) populations during homeostasis, representing less than 1% of circulating leukocytes (44). Due to meager levels of these cells, it has been difficult to study the functions of basophils *in vivo*. However, a number of recent studies have underscored the importance of basophils during the immune response.

Hematopoietic progenitors in bone marrow produce granulocytes such as basophils (5). In response to certain infections, basophils are recruited to the site of invasion and participate in inflammatory attacks on pathogens (45). Basophils express high levels of FcεRI, the receptor for Fc regions of IgE antibodies. Cross-linkage of antigen-specific IgE on the surface of basophils activates the cell causing degranulation and the release of preformed mediators such as histamine. Activation also causes basophils to produce a number of cytokines such as IL-4 (46).

There has been a renewed interest in basophils and their role in immunity because of their ability to produce IL-4 (47, 48). Pioneers in basophil biology have recently determined that basophil derived IL-4 is especially important in Th2 differentiation. For example, Sokol *et al.* (2008) showed basophils exposed to protease allergens can travel to the draining lymph node and provide the initial signal to promote Th2 differentiation *in vivo,* likely through their production of IL-4 and other similarly acting cytokines (49). In fact, mounting evidence indicates basophils and their production of IL-4 are directly responsible for producing Th2 responses (44, 46, 48, 50, 51).

Recent studies indicate basophils can promote Th2 immune responses independent of IL-4 production alone. For example, investigators have demonstrated that basophils, rather than dendritic cells, can serve as APCs and provide the essential cytokines which drive Th2 responses to the cysteine protease papain (49). Two additional studies have also suggested basophils can function as APCs (52, 53), which until recently, was an unknown function of this cell.

The function of IgE cross-linked basophils can be increased by a variety of other stimuli including cytokines (54, 55), proteases (49), helminth products (56, 57), and possibly toll-like receptors ligands and complement proteins (58). However, the most powerful stimulus

which synergizes with activated basophils appears to be IL-3 (46), which has been shown to be indispensable for increased basophil functionality and proliferation during certain infections. For instance, Lantz *et al.* (1998) showed at physiological conditions basophil levels in IL-3 +/+ and IL-3 -/- mice were approximately equal indicating IL-3 is not required for producing baseline levels of basophils. However, IL-3 +/+ mice produce markedly higher levels of basophils in bone marrow and blood compared to IL-3 -/- mice following infection with the nematodes *Stronglyoides venezuelensis* and *Nippostrongylus brasiliensis* (24, 59). Importantly, these investigators also show basophil levels in nematode-infected IL-3 -/- mice were essentially the same as basophil levels seen in uninfected mice. These studies indicate that, although IL-3 is not required for producing baseline levels of basophils, it is essential for practically all increases in basophils in response to gastrointestinal nematodes.

More recently, Lantz *et al.* (2008) showed that IL-3 stimulation induces activated basophils to upregulate their production of IL-4 (24). As stated above, IL-4 is the primary cytokine responsible for driving Th2 development, the susceptible response to CL. Nevertheless, it has not been determined if IL-3 stimulated-basophil-derived IL-4 is responsible for Th2 responses characteristic of *L. major*-infected susceptible BALB/c mice.

Project goals

A number of studies have reported that IL-3 production correlates with susceptibility of BALB/c mice to infection with *L. major* and that IL-3 can promote basophil expansion and enhance basophil cytokine production. However, the precise role of IL-3, and IL-4-producing basophils, in CL has yet to be addressed. We propose to take advantage of exciting opportunities to analyze the role of IL-3 and basophils in a mouse model of CL. Specifically, we will use BALB/c IL-3-deficient (IL-3 -/-) mice and their wild type counterparts (IL-3 +/+ mice), to test two major but related hypotheses: 1) that endogenous

IL-3 significantly promotes the susceptibility of BALB/c mice to *Leishmania major* infection as reflected by measures of lesion size and parasite burden, and 2) that *Leishmania major* infection induces IL-3-dependent increases in basophils.

The specific aims of this project are as follows: (start revision Rajeev here)

Aim 1 - Assess the extent to which IL-3 influences the course and outcome of disease in Leishmania major-infected BALB/c mice. We used BALB/c IL-3 -/- and +/+ mice infected with different inocula of *L. major* to test the hypothesis that endogenous IL-3 contributes to the development of cutaneous lesions and the high parasite load characteristic of susceptible BALB/c mice.

Aim 2 - Assess the extent to which basophils influence disease susceptibility in Leishmania major-infected BALB/c mice. We employed IL-3 +/+ mice to test the hypotheses: 1) that *L. major* induces increases in basophil numbers in bone marrow, and 2) that *L. major* infection and associated IL-3 production induces basophil migration to the draining lymph nodes in IL-3 +/+ mice.

Materials and Methods

Mice

The production of IL-3 -/- BALB/c mice, and many of the phenotypic characteristics of

these mice, has been described in detail (60-62) and were graciously provided by Dr. Chris

Lantz. Wild type BALB/c mice were purchased from Jackson laboratories and used as IL-3

+/+ mice. For the present studies, mice were 8-12 weeks of age at the beginning of the

experiment. For individual experiments, IL-3 +/+ and IL-3 -/- mice of the same sex and

approximately the same age were used (n = 3 – 5) and experiments were repeated up to

three times. The genotypes of randomly selected mice were verified by PCR essentially as

previously described (61) (Appendix Fig. 14). Mice were housed in microisolater cages in the

Department of Biology's animal facility under a 12h light-dark cycle and were maintained

and used in accordance with James Madison University's Institutional Animal Care

guidelines.

Parasites and infection

Leishmania major LmFV1, a virulent clonal derivative of the Friedlin line

(MHOM/IL/80/Friedlin) was kindly provided by Dr. David Sacks (National Institutes of

Health). *L. major* promastigotes were cultured in medium 199 containing Hank's salts and L-

glutamine (M-199) (Sigma-Aldrich) with 0.1 mM adenine (in 50 mM HEPES), 100 U/mL

Penicillin, 100 μg/mL Streptomycin (Sigma), 5 μg/mL hemin (in 50% (v/v)

triethanolamine) (Fluka, Aldrich), and 20% heat inactivated fetal calf serum (FCS) at a pH of

approximately 7.15 (cM-199).

cM-199 facilitates the process of metacyclogenesis allowing parasites to develop from a

dividing, noninfective stage to a nondividing, infective stage (Appendix Fig. 15). During the

initial days of culture (days 1-2), parasites grow at an exponential rate and are said to be in logarithmic phase until day 3-4 when growth starts to plateau. Once growth levels off, parasites cultures enter the stationary phase, which tends to yield a sufficient number of metacyclic promastigotes for infecting mice (Appendix Fig. 16).

Infective stage metacyclic promastigotes of *L. major* were isolated from stationary cultures (4-6 day old) using negative selection with peanut agglutinin (Vector Laboratory Inc.) as previously described (63). 1.0×10^4- 2.5×10^4 infective stage parasites in 10 or 15 μL were then injected into the dermis of either the right or left ear of BALB/c mice (Appendix Fig. 17). The uninfected ear was used as a control and injected with an equal volume of sterile Phosphate Buffered Saline (PBS). Images of uninfected and *L. major*-infected ears are shown in Appendix Figure 18. All injections were given with 31 gauge hypodermic needles fitted on a 0.3 mL syringes.

The course of *L. major* infection was monitored at weekly intervals by measuring the thickness of infected ears with a Mitutoyo digital micrometer. Lesion sizes were expressed as the change in thickness by measuring the infected ears and subtracting the thickness of the ear prior to infection (baseline). Photographs were also taken of representative mice from IL-3 +/+ and IL-3 -/- groups at different time points during infection.

Parasite Quantification

To determine parasite load in *L. major* infected mice, a serial dilution assay was performed *in vitro* essentially as previously described (63). Infected ear samples were collected at different time points post-infection and weighed. Using sterile forceps, infected ears were separated into ventral and dorsal sheets and placed into sterile 35mm petri dishes containing Dulbecco's Modified Eagle Medium (DMEM), 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.5 mg/mL Liberase TL (Roche). Infected ear sheets were digested at

$37°C$ at 5% CO_2 for approximately three hours. After incubation, ear sheets were removed and placed in a clean sterile 35 mm petri dish and cut into small pieces using a sterile scalpel. Ear tissues were then transferred to a microfuge tube containing 100 μL CM-199 and homogenized with a Teflon coated pestle. After homogenization, 0.9 mL of CM-199 was added and the homogenates were strained twice through a 70 μm cell strainer. A 125 μL sample of the resulting single cell suspension was added to one well of a 96-well plate containing 250 μL of cM-199, resulting in a 1:3 dilution. Serial dilutions (1:3) were then performed on the 96-well plate to extinction. Each dilution was then plated in duplicate in 96-well plates containing slanted blood agar and incubated for 7 days at 26°C. After incubation, each dilution was monitored for parasite growth via an inverted microscope. The reciprocal of the largest dilution at which promastigotes could be detected in duplicate samples was then averaged and reported for each mouse.

Parasitic load was also determined using quantitative, relative real-time PCR to detect different levels of *L. major* DNA in IL-3 +/+ and IL-3 -/- ears essentially as previously described (64). Briefly, infected and uninfected ears of IL-3 +/+ and IL-3 -/- mice were collected, snap frozen in liquid nitrogen, and the DNA extracted using GenElute Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich). Each sample of extracted DNA (10 ng) was subjected to real-time PCR in triplicate using Platinum SYBR Green qPCR SuperMix-UDG cocktail (invitrogen) essentially according to manufacturer's protocol and a Bio-Rad CFX96 Real-Time PCR Detection System. Amplification of *Leishmania*-specific DNA was performed using the previously described primers, forward: 5-CCTATTTTACACCAACCCCCAGT-3 (JW11); and reverse: 5-GGGTAGGGGCGTTC TGCGAAA-3 (JW12) (Intergrated DNA Technologies) (64, 65). DNA was initially denatured for a single cycle at 95°C. After the initial denaturation step, DNA was denatured

at 95°C for 5 sec followed by a combined annealing/extension step at 58°C for 5 sec. Denaturation and annealing/extension steps were repeated 39 times and fluorescence intensity was measured at the end of each cycle. Relative amounts of parasite DNA present in *L. major*- infected IL-3 +/+ and IL-3 -/- ears is reported as the threshold cycle (C_T), at which there was a positive signal for parasite DNA over the background. Samples from uninfected IL-3 +/+ and IL-3 -/- mice ears were used as controls to ensure primers were specific for *L. major* DNA.

Quantification of lymphocytes by flow cytometry

LN cells were collected from *L. major* infected IL-3 +/+ and IL-3 -/- mice to identify $CD4^+$ and B cell populations using an Accui C6 flow cytometer. All samples were treated with purified anti-mouse monoclonal antibody (mAb) CD16/CD32 (clone 2.4G2) (10 µg/mL) for 10 min to prevent antibody binding to $Fc_\gamma RII/III$ prior to staining cells. All staining and incubation steps were performed at 4 °C. To identify $CD4^+$ cells, samples were stained with Fluorescein isothiocyanate (FITC) anti-mouse CD4 mAb (clone L3T4) (5 µg/mL). To identify B cell populations, LN cells were stained with and Phycoerythrin (PE) anti-mouse CD45R mAb (clone B220) (4 µg/mL). Prior to examining stained cells, lymphocytes were initially identified by their characteristic forward (FSC) and side scatter (SSC) properties.

Quantification of basophils by flow cytometry

LN and bone marrow samples were also evaluated via flow cytometry for the presence of basophils. Bone marrow-derived basophils were identified based on their expression of FcεRI by incubation with mouse IgE (30 min) followed by staining with FITC anti-mouse IgE mAb (clone R35-72) (10 µg/mL). Because B cells also express low affinity IgE Fc

receptors (CD23), they were distinguished from basophils by staining with PE CD45R mAb (clone B220) (4 μg/mL). To identify basophils in LN samples, cells were first treated with purified anti-mouse CD23 mAb (clone B3B4) to block low affinity Fc IgE receptors on B cells. Samples were then incubated with ascites IgE (30 min) prior to staining with FITC anti-mouse IgE mAb (clone R35-72) (10 μg/mL) and PE anti-mouse CD49b mAb (clone DX5) (4 μg/mL). All flow cytometric data was analyzed on the Accuri C6 using CFLOW Plus Software.

Statistical analysis

Values of $p \leq 0.05$ were considered statistically significant as determined by an unpaired, two tailed Student's *t* test unless otherwise noted.

Results

IL-3 influences cutaneous lesion development in L. major-infected mice

To evaluate the functions of IL-3 in CL, IL-3 +/+ and IL-3 -/- mice were infected with *L. major* to determine the ability of this cytokine to influence the size of developing lesions. The thickness of *L. major*-infected IL-3 +/+ and IL-3 -/- mice ears were measured weekly over a course of ten weeks. As expected, BALB/c IL-3 +/+ mice developed noticeable lesions which appeared approximately three weeks post-infection. By four weeks, IL-3 +/+ lesions were readily apparent and displayed visual inflammation accompanied by ulceration that persisted throughout the 10 week infection. Interestingly, IL-3 -/- mice developed lesions which were barely detectable at three weeks and inflammation was not noticeable until approximately six weeks post-infection. Overall, IL-3 +/+ mice developed significantly thicker ears compared to IL-3 -/- mice throughout the 10 week infection (Fig. 4). The degree of inflammation was also visually different in IL-3 +/+ and IL-3 -/- mice (Fig. 5).

Lesion size has been shown to be dependent on dose of parasites used to infect mice (66-68). Therefore, infections were repeated with lower parasite inocula (1.5×10^4 or 1.0×10^4) to determine if differences in lesions among IL-3 +/+ and IL-3 -/- mice could still be observed when infecting with fewer parasites. Comparison of lesion size between the two genotypes showed IL-3 +/+ mice infected with 1.5×10^4 metacyclic promastigotes displayed lesions which were thicker compared to similarly infected IL-3 -/- mice (Fig. 6A). Likewise, similar differences were seen in lesion thickness when mice were infected with 1.0×10^4 parasites (Fig. 6B). Regardless of infectious dose, IL-3 +/+ mice lesions displayed an advanced level of ulceration and appeared to be more inflamed compared to IL-3 -/- mice at similar time points of infection. Similar to mice shown in Fig. 4, statistically significant differences in lesion size became apparent at three to four weeks post-infection. This

Figure 4 Infected ear thickness from IL-3 +/+ and IL-3 -/- mice. Both BALB/c IL-3 +/+ and IL-3 -/- mice were infected into the ear dermis with $2.0x10^4$ metacyclic promastigotes in 15uL inoculums. Contralateral ears were injected with 15uL of sterile PBS. Thickness of infected ears were measured weekly for 10 weeks. Data represent the mean ± SEM (n = 4 mice/group). Statistical significance was determined by an unpaired, two tailed student's t-test. P value was ≤ 0.05 at weeks 4 – 9. P value = 0.056 at 10 weeks.
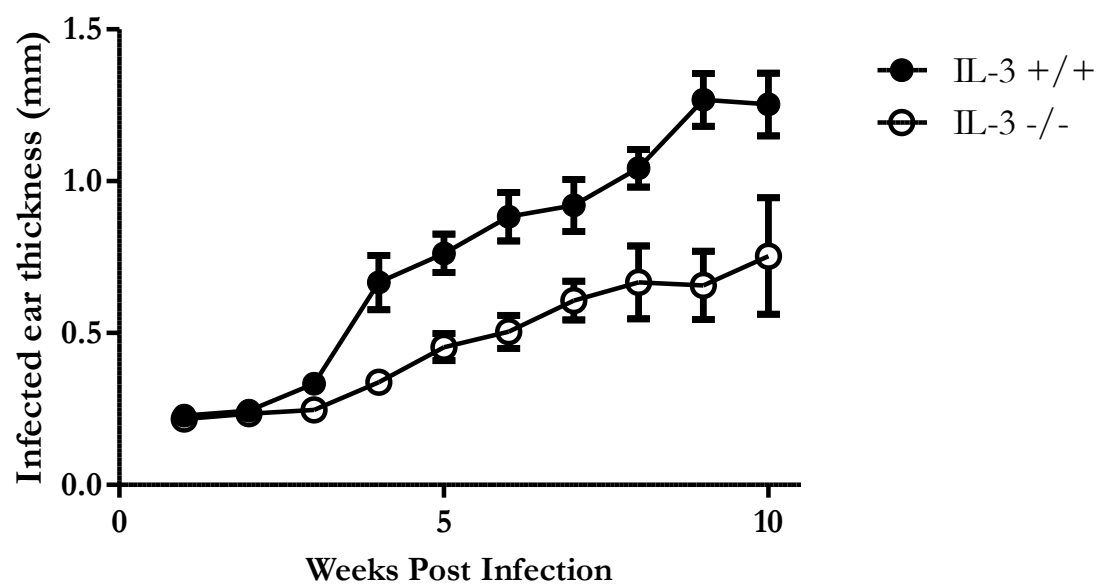
Figure 5 *L. major* infected IL-3 +/+ (A) and IL-3 -/- (B) mice 6 weeks post-infection with $2.0\text{x}10^{5}$ metacyclic promastigotes.
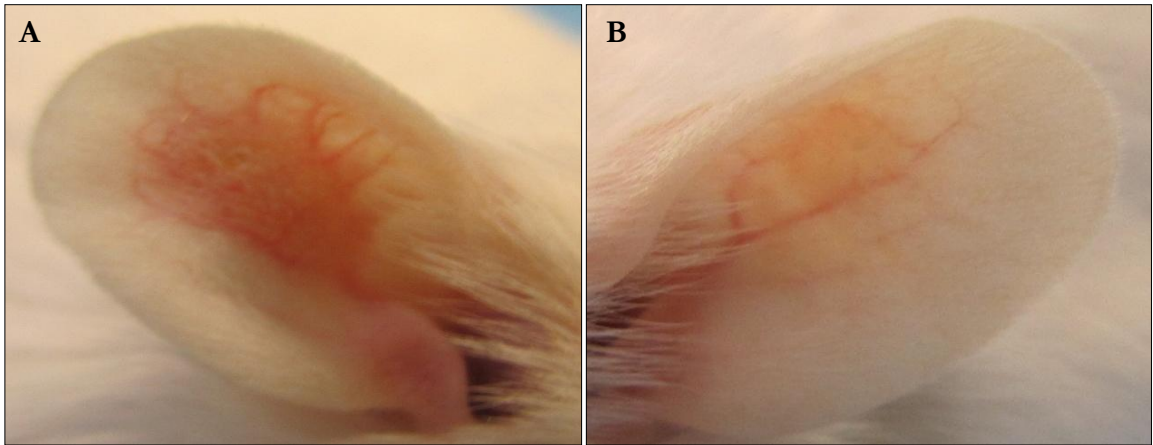
Figure 6 Lesion development in BALB/c IL-3 +/+ and IL-3 -/- mice infected with 1.5 x 10^4 (A) or 1.0 x 10^4 (B) infective stage *L. major* metacyclic promastigotes. *L. major* promastigotes were injected subcutaneously into the dermis of either the right or left ear. The contralateral ear was injected with equal amounts of sterile PBS. Data represent mean ± SEM (n = 4 or 5 mice/group). Statistical significance was determined at four weeks post-infection by an unpaired, two-tailed Student's t-test. Asterisk (*) indicates a p value ≤ 0.05 versus corresponding values for mice of the other genotype.

A
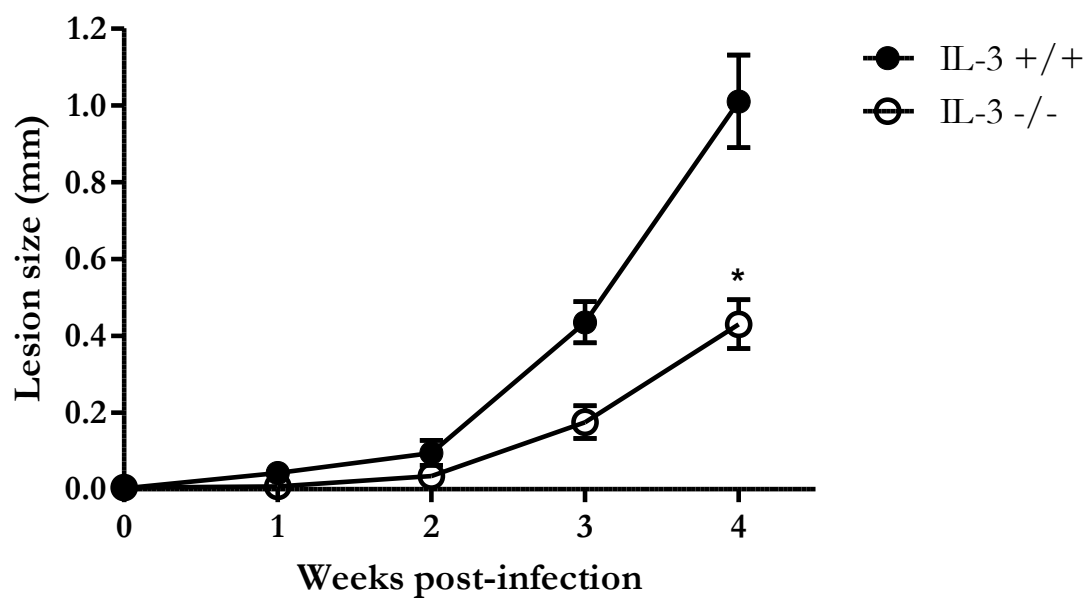
B
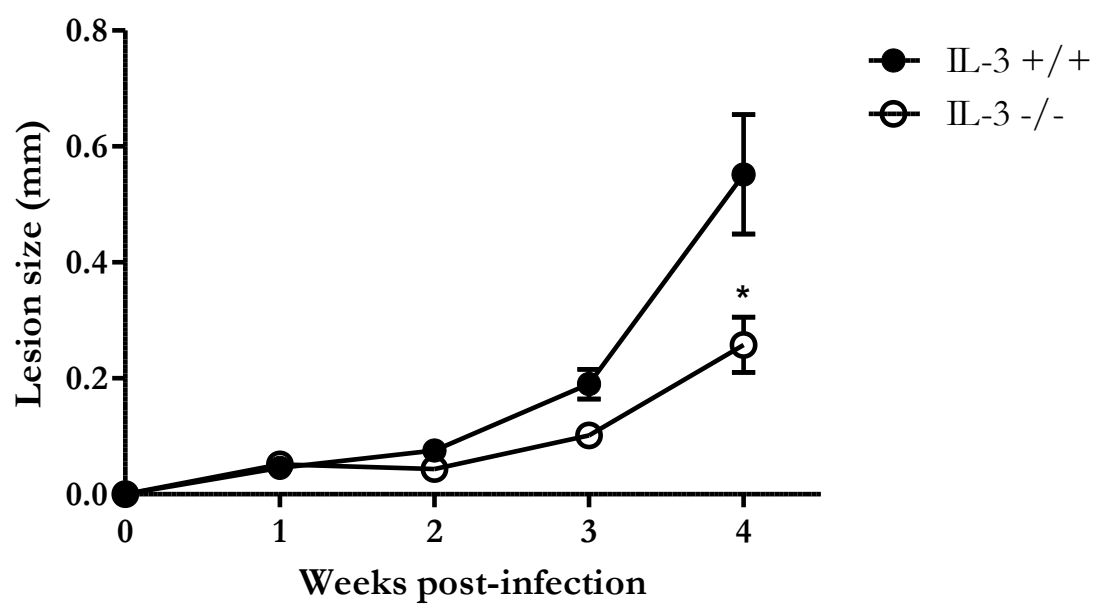
suggests that irrespective of infectious dose, IL-3 influences the development of *L. major*-induced cutaneous lesions.

These data indicate IL-3 influences lesion size starting at three weeks post-infection and that these differences are maintained as long as 10 weeks post-infection. We next wanted to see if the effects of IL-3 were evident at greater than 10 weeks post-infection. For this experiment, mice were infected with 1.0×10^4 parasites. Fewer metacyclic promastigotes were used in this experiment because in BALB/c mice given higher doses, *L. major* becomes lethal through its ability to visceralize and infect other organs such as the spleen and liver (26).

These studies found that differences seen in lesion sizes from four to 10 weeks post-infection could also be detected in mice infected for four months (Fig. 7). In fact, lesions in IL-3 +/+ mice were on average twice the size of those observed in IL-3 -/- mice. Consistent with data from shorter-term experiments, IL-3 +/+ mice appeared to have more necrosis and inflammation at the site of infection. Collectively, these data suggests IL-3 can influence the degree of lesion development at both high and low parasite doses and continues to affect lesion inflammation in more chronically infected mice.

Surprisingly, two of the four IL-3 -/- mice infected for four months appeared to clear the infection. These two mice had significant lesions at four weeks post-infection. However, by four months post-infection lesion size had essentially returned to baseline levels and very little inflammation could be detected visually (Fig. 8). The ability of some IL-3-/- mice to apparently clear their infection is an exciting finding because it may suggest in the absence of IL-3, mice possibly develop a more protective Th1-dominated immune response.

IL-3 influences parasitic load L. major-infected mice

Past research has shown lesion size in *L. major*-infected mice does not always correlate with numbers of parasites present in infected tissue. For example, some investigators have

Figure 7 Ear thickness of uninfected and *L.major*-infected BALB/c IL-3 +/+ and IL-3 -/- mice. Measurements were taken four months post-infection with $1.0x10^4$ metacyclic promastigotes in 10uL inoculums. Data represent mean $\pm$ SEM (n = 4 mice/group). Statistical significance was determined by an unpaired, two tailed Student's t-test. Double asterisk (**) indicates a p value ≤ 0.01 compared to mice of the other genotype.
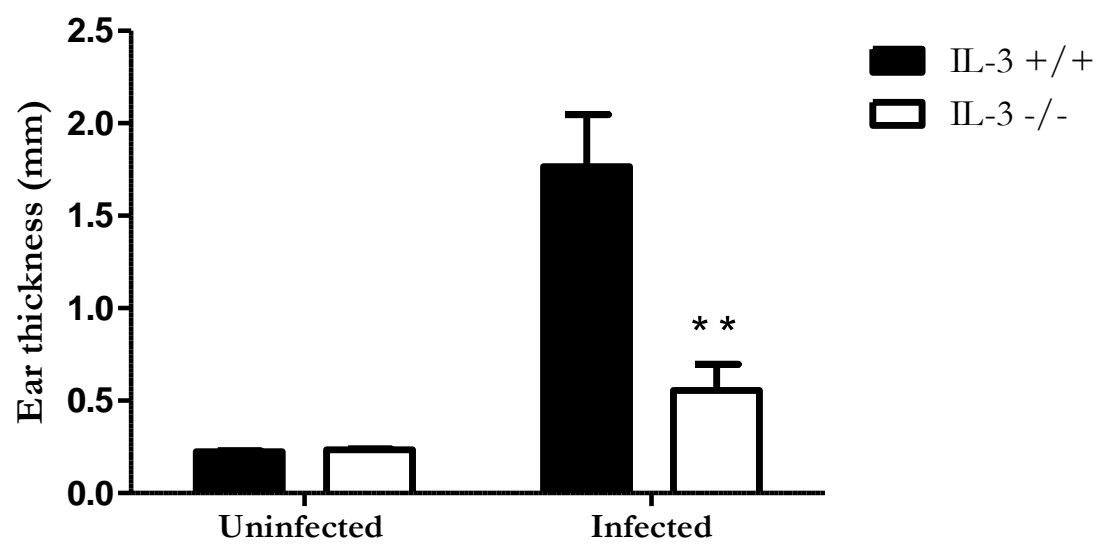
Figure 8 Image of IL-3 -/- mouse ear four months following infection with 1.0 x 10^4 metacyclic promastigotes. Minimal inflammation was detectable in these infected ears which appeared visually similar to control uninfected ears (see Fig. 18).

reported increased lesion size is a function of increased parasite load (69). However, others have shown maximum parasite burden occurs prior to the time infected mice present greatest lesion size (70). Therefore, it was next determined if differences seen in lesion sizes of IL-3 +/+ and IL-3 -/- mice correlated with differences in parasite burden.

The standard procedure for estimating parasite load during *L. major* infection is a cell culture assay in which serial dilutions of infected ear tissue are cultured in blood agar. However, new protocols which utilize real-time PCR (qPCR) and the ability to identify *L. major* DNA have been described which are less laborious and could prove to be much more accurate. Therefore, both limited dilution culture of parasites from infected tissue and relative qPCR were used to estimate parasite burden in infected groups of mice.
Limited dilution culture assays indicated IL-3 +/+ mice had substantially more *L. major* parasites per milligram of tissue at four weeks post-infection compared to similarly infected IL-3 -/- mice (Fig. 9). Furthermore, qPCR results indicated samples from *L. major*-infected IL-3 +/+ mice had significantly lower C_T values four months post-infection than IL-3 -/- mice (Fig. 10). C_T values indicate the cycle at which a positive signal for target DNA is detected. Therefore, lower C_T values in IL-3 +/+ mice indicates they had more *L. major* DNA at the infection site, suggesting chronically infected IL-3 +/+ mice have higher parasite burdens compared to similarly infected IL-3 -/- mice. There was also a general trend in these experiments showing mice which had the thickest lesions also had the highest concentration of parasites at the site of infection, regardless of genotype. This suggests there is a positive correlation between lesion thickness and concentration of parasites in infected tissue. Additionally, the two IL-3 -/- mice that appeared to clear the chronic infection showed only slightly higher levels of *L. major* DNA compared to uninfected controls.

IL-3 influences lymphocyte populations in L. major-infected mice

Figure 9 Number of parasites per mg of infected ear tissue. *L. major*-infected BALB/c IL-3 +/+ and IL-3 -/- mice ears were collected and weighed 4 weeks following infection with $1.0x10^4$ metacyclic promastigotes. Cells harboring parasites were extracted from infected ears and serially diluted as described in the materials and methods. The reciprocal of the largest dilution at which promastigotes could be detected in duplicate assays was then averaged and reported as the mean ± SEM (n=5 mice/group). Statistical significance was determined by an unpaired, two tailed Student's t-test. p value = 0.0503 for infected IL-3 +/+ and IL-3 -/- mice. The data shown are from a single experiment that is representative of two separate experiments.
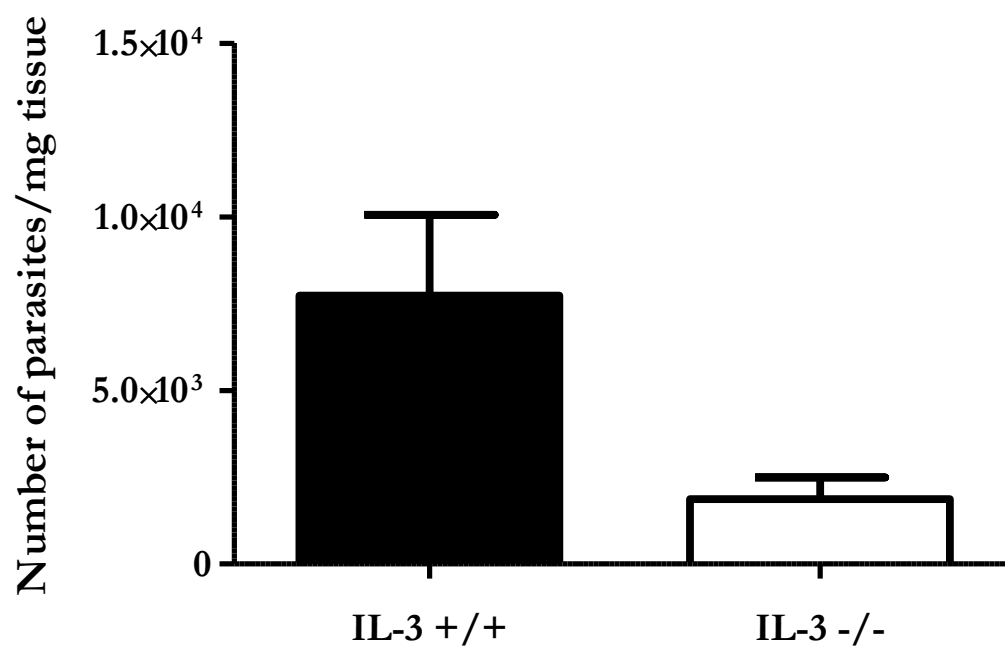
Figure 10 Parasite load in *L. major*-infected BALB/c IL-3 +/+ and IL-3 -/- mice ears 4 months following infection with 1.0×10^4 metacyclic promastigotes. Infected ears (n = 4 mice/group) were collected and analyzed for *L. major* DNA via qPCR as described in the materials and methods. One uninfected ear from each group was also collected to serve as a negative control. Data represents mean threshold cycle (C_T) at which each sample was positive for *L. major* DNA. Statistical significance was determined by an unpaired, two tailed Student's t-test. Asterisk (*) indicates a p value ≤ 0.05 versus corresponding values for mice of the other genotype.
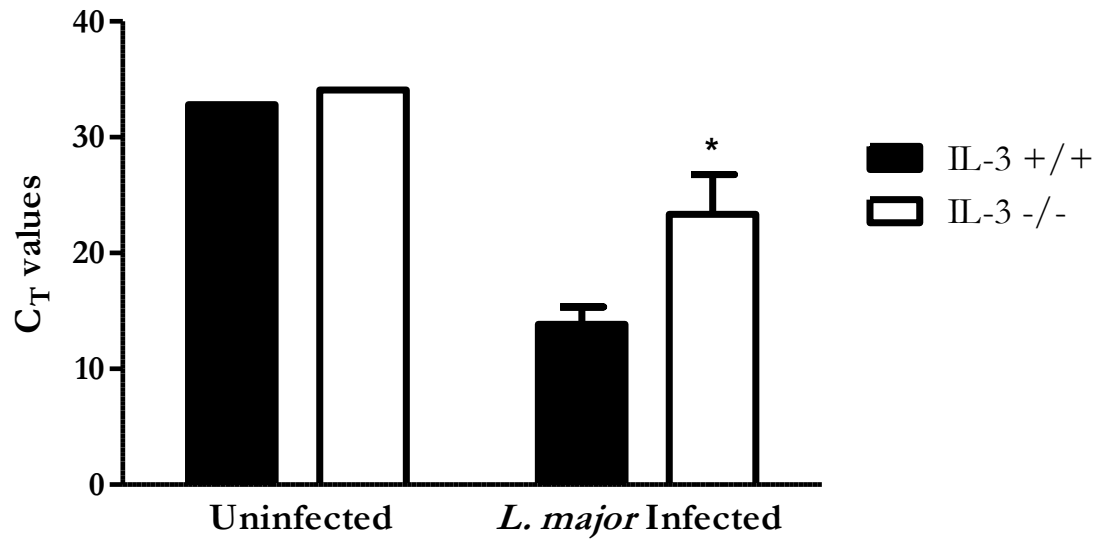
Generation of adaptive immune responses against *Leishmania* parasites coincides with the expansion of certain cell populations in the LNs of infected organisms. The sub-populations of lymphocytes which undergo expansion influence if a host is resistant or susceptible to this infection. Therefore, the effect of IL-3 on lymphocyte populations was monitored in LNs of uninfected and *L. major*-infected IL-3 +/+ and IL-3 -/- mice. Levels of $CD4^+$ T cells and B cells were identified in auricular LNs by flow cytometry from *L. major*-infected mice at four weeks post-infection and in uninfected mice.

These analyses revealed essentially no difference in the levels of $CD4^+$ T cells and B cells in uninfected IL-3 +/+ and IL-3 -/- mice. However, four weeks after infection with 1.0 x 10^4 metacyclic promastigotes, IL-3 +/+ mice had a significantly higher percentage of B cells than similarly infected IL-3 -/- mice (Fig. 11). Interestingly, LNs from IL-3 +/+ mice also contained a substantially lower percentage of $CD4^+$ T cells compared to IL-3 -/- mice (Fig. 11). These data indicate IL-3 may promote the expansion of B cells and inhibit T helper cell development. This effect on B and T cells indicates that IL-3 could be abrogating a protective immune response, although it is unclear if this effect is direct or indirect.

IL-3 regulation of basophil development in L. major-infected mice

Activated basophils have gained interest from investigators studying the immune system because of their recently discovered ability to promote T_H2 responses by producing IL-4 (50, 71). Furthermore, studies have demonstrated IL-3-stimulated basophils can undergo increased proliferation and IL-4 production in response to certain infections (24, 59). As previously mentioned, susceptibility to CL is promoted by IL-4-driven Th2 host responses. However, to our knowledge no one has examined the effects of IL-3 or basophils during *Leishmania* infection. Therefore, basophil levels were monitored in infected mice to see if

Figure 11 Percent of $CD4^+$ T cells and B cells in LNs of uninfected and *L. major*-infected mice (A). Uninfected and *L. major*-infected LNs were collected from IL-3 +/+ and IL-3 -/- mice at 4 weeks post-infection with 1.0×10^4 metacyclic promastigotes and analyzed via flow cytometry. (B, C) Dot plots representative of data from Fig. 3.10A showing $CD4^+$ and $B220^+$ cells from uninfected (B) (n = 3) and infected (C) (n = 5) LN populations. The data shown are from a single experiment that is representative of two separate experiments. Statistical significance shown in Fig 3.10A was determined by an unpaired, two tailed Student's t-test. Asterisk (*) indicates a p value $\leq$ 0.05 versus corresponding values for mice of the other genotype.
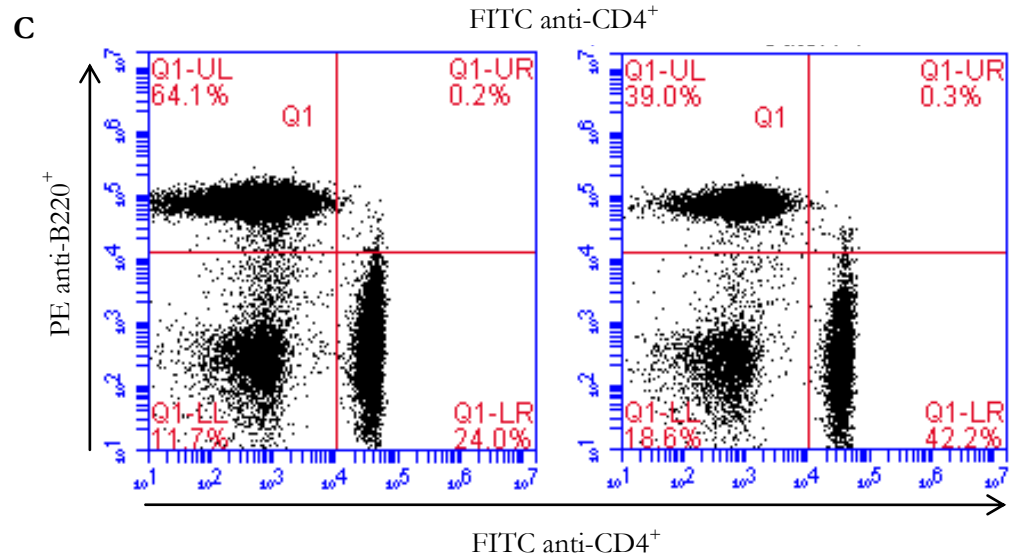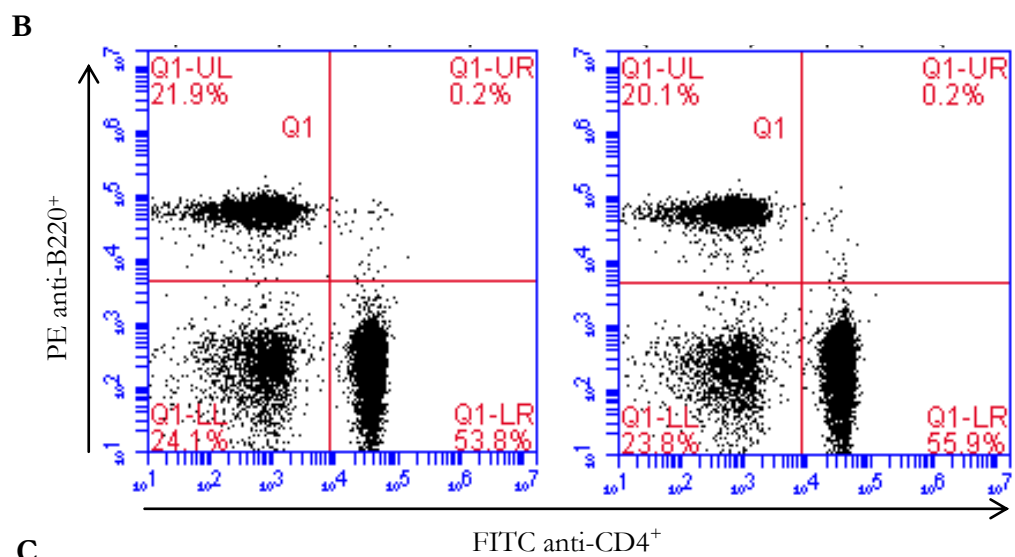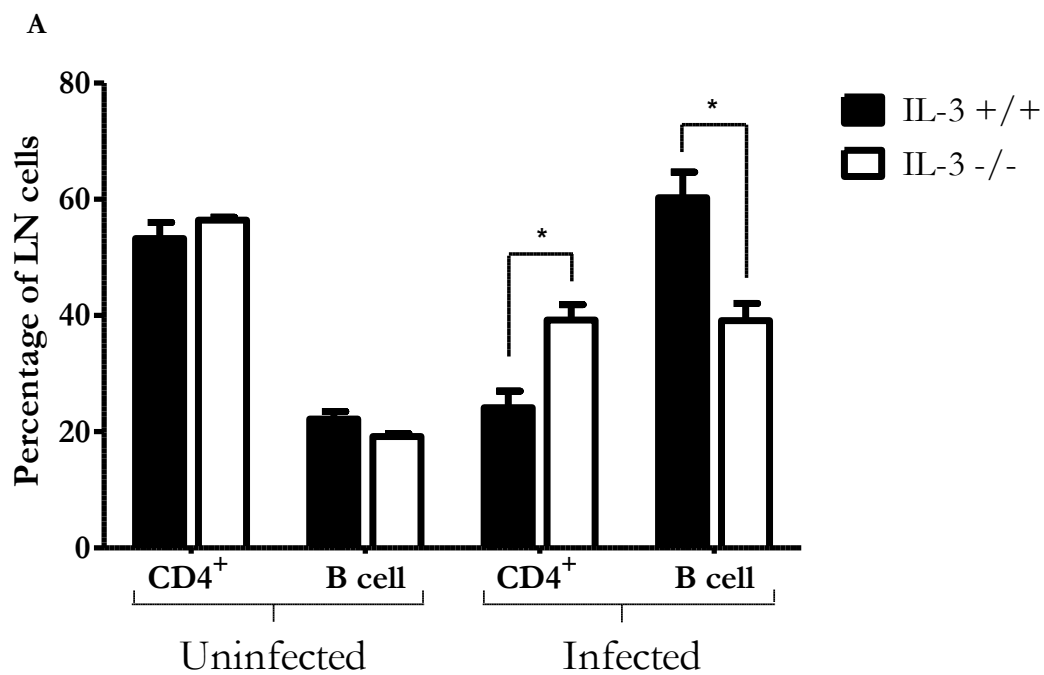
increases could be detected in response to *L. major* parasites. Initially, these studies were only performed with IL-3 +/+ mice because increases in basophils were not expected in IL-3 -/- mice (24, 50). Therefore, if no increases in basophil levels were detected in IL-3 +/+ mice, it is unlikely that increases would be seen in IL-3 -/- mice.

As expected, results from these experiments indicate basophil numbers in bone marrow samples from uninfected BALB/c IL-3 +/+ mice were less than 1 percent, typical for mice under baseline physiological conditions (59). Interestingly, after infecting mice with 2.0×10^4 metacyclic promastigotes, basophil levels in the bone marrow remained below one percent and were essentially the same as compared to uninfected mice. This experiment indicated infection with a relatively high dose of *Leishmania* parasites induces no increases in basophil numbers in BALB/c mice at 5 weeks post-infection (Fig. 12).

Basophil activity was also evaluated in other tissues of *L. major*-infected BALB/c IL-3 +/+ mice. LNs, which are typically devoid of basophils, were studied because any observed increase in basophil numbers might suggest they migrate to lymphoid tissue and contribute to IL-4 dependent immune responses. Draining LNs were collected from mice infected with 1.0×10^4 metacyclic at days four, nine, and four weeks post-infection and basophils were detected by flow cytometry. However, detectable levels of basophils were not observed in LN samples from BALB/c IL-3 +/+ mice at any time point evaluated (Fig. 13); suggesting *Leishmania* infection may not induce basophil proliferation and/or basophil migration to LNs.

Figure 12 Basophil levels in uninfected and infected BALB/c IL-3 +/+ mice injected with $2.0\text{x}10^4$ metacyclic promastigotes (A). Bone marrow samples were collected 5 weeks post-infection from uninfected (n=3) and infected (n=4) BALB/c IL-3 +/+ mice and analyzed for basophil levels via flow cytometry. (B, C) Dot plots representative of data from Fig 3.11 A showing PE B220$^+$ and FITC IgE$^+$ cells from bone marrow samples of uninfected (B) and infected (C) mice. Basophils were considered FITC anti-IgE$^+$ and PE B220$^-$.
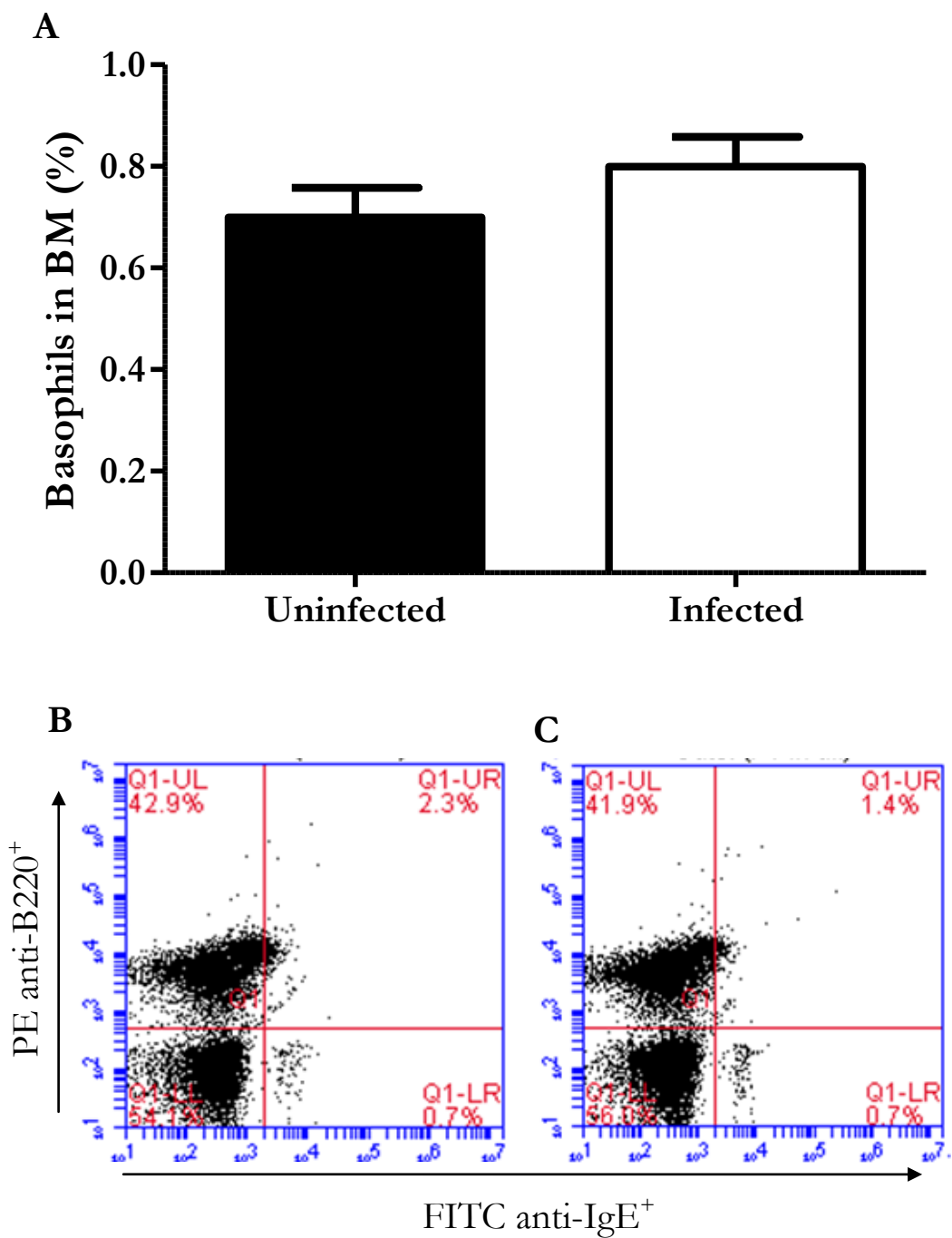
Figure 13 Basophil levels in LNs from BALB/c IL-3 +/+ mice infected with $1.0x10^4$ *L. major* metacyclic promastigotes. LN cells were collected at days 4 (A), 9 (B), and 4 weeks (C) post-infection and analyzed via flow cytometry. No IgE^+, $CD49b^+$ basophils were observed in LNs from any mouse at the time points evaluated (n = 3-5).

48
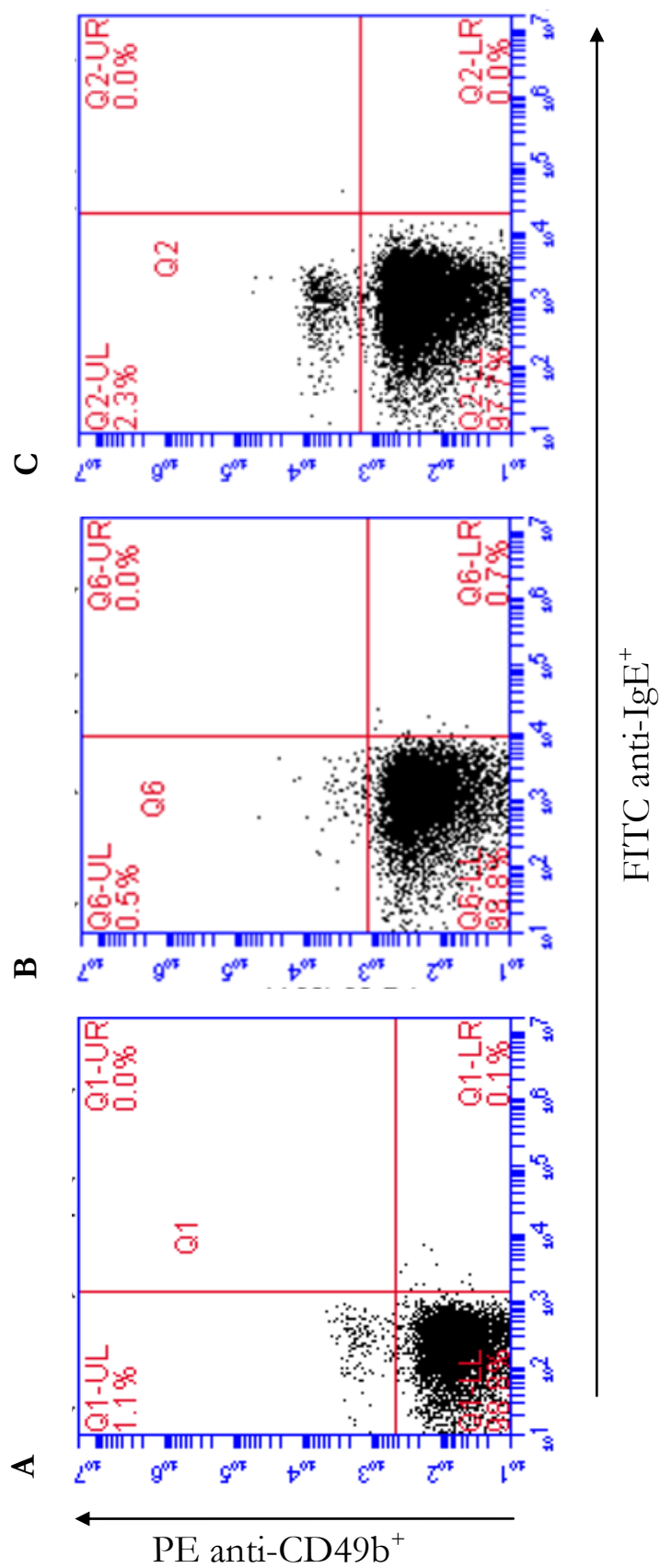
Discussion

A limited number of *in vitro* studies have suggested that IL-3 influences the course of CL, however, to our knowledge no studies have shown an *in vivo* role for IL-3 during this disease. BALB/c WT (IL-3 +/+) and IL-3 deficient (IL-3 -/-) mice were subcutaneously injected with *L. major* promastigotes in one ear and the clinical course of infection was monitored for up to four months. In the absence of IL-3, normally susceptible BALB/c mice infected with 2.0×10^4 metacyclic promastigotes showed less inflammation based on lesion size compared to BALB/c IL-3 +/+ mice. These differences were especially evident throughout the course of a 10 week infection (Fig. 4).

Significant differences in infected ear sizes of IL-3 +/+ and IL-3 -/- mice examined at four weeks post-infection were also noted when mice were infected with smaller parasite doses (Fig. 6). Likewise, IL-3 -/- mice infected for four months also showed smaller lesions compared to similarly infected IL-3 +/+ mice (Fig. 7). However, it is important to mention the mice used in the four month experiment became co-infected with *Syphacia obvelata* (mouse pinworms). Lesion sizes among co-infected IL-3 +/+ and IL-3 -/- were not significantly different (data not shown), likely due to a strong Th2 type immune response produced by BALB/c mice in order to clear the pin worm infection (72). However, after pinworm treatment, significant differences in lesions sizes of IL-3 +/+ and IL-3 -/- mice were seen four months post-infection(Fig 7). No evidence of pinworm infection was observed with other experiments reported in this study.

Significant differences in lesion sizes among the two genotypes of mice suggest IL-3 operates *in vivo* during the immune response to *Leishmania* parasites, a previously unknown function of this cytokine. Thus, based on measurements of lesion size alone, it appears IL-3

exacerbates disease. However, a number of reports suggest that lesion size does not necessarily correlate with the number of parasites present at the inflammatory site (reviewed by (63)).

The sizes of inflamed lesions that develop during the course of *L. major* infection can be attributed to a number of factors. First, lesion size can be influenced by the number of parasites present at the site of infection, with a higher number of parasites leading to larger lesions. One could also argue lesion size is regulated by the potency of the host organism's immune response. For example, if an organism makes a robust immune response, many effector cells become activated and migrate to the site of infection where they can initiate pro-inflammatory activities resulting in increased lesion sizes. Alternatively, weak immune responses results in fewer or inefficient activation of cells causing less cell migration to the infection site resulting in smaller lesions. Therefore, observing larger lesions in IL-3 +/+ mice could be caused either by a higher parasite burden or a more robust immune response.

To determine if larger lesions present in IL-3 +/+ mice were caused by a higher parasite burden, *L. major* infected IL-3 +/+ and IL-3 -/- mice were sacrificed four weeks post-infection and the parasite load of each individual lesion that developed in the two genotypes of mice were examined (Fig. 9). This experiment was repeated twice using serial dilutions of cells from infected ear tissue. Both of these experiments showed IL-3 +/+ mice maintain a higher parasite burden than similarly infected IL-3 -/- mice indicating their increased lesion size is likely caused by a higher parasite burden. Higher parasitic loads in IL-3 +/+ mice are likely caused by an inability to effectively eliminate the parasites suggesting IL-3 may inhibit leishmaniacidal activities of the immune system. Taken together, the experiments that examined lesion size and parasite burden suggest that IL-3 is detrimental to the host during *Leishmania* infection.

Protocols for the diagnosis and evaluation of parasite burden have been improved as research with *Leishmania* progresses. Traditional protocols call for separation and enzyme degradation of infected tissues into a single cell suspension. These single cell suspensions are then serially diluted and incubated with nutrient rich medium which promotes promastigote growth. Each dilution is monitored for parasites approximately seven to ten days after plating, and the reciprocal of the highest dilution at which parasites are present is considered to be the parasite burden. Determining parasite load with such protocols is not accurate because they only enable an estimate of the true parasite burden. This method only provides an estimate because the data reported are dilution factors instead of absolute parasite numbers. Due to the drawbacks inherent with this method, a fairly new protocol utilizing real-time PCR was performed to validate the data on parasite burden. According to previous work, this technique is extremely sensitive and can detect minute levels of *L. major* DNA equivalent to 0.1 parasites (65). Increased assay sensitivity is ideal, particularly when attempting to diagnose or quantitate levels of parasites in mildly-infected animals. Therefore, quantitative PCR was initially employed to determine parasite load in mice infected for four months (Fig. 10). This experiment was chosen to determine if IL-3 -/- mice that had seemingly cleared their parasite infection still harbored *L. major* DNA. These data showed that IL-3 +/+ mice had markedly higher C_T values when compared to IL-3 -/- counterparts indicating that infected tissue from IL-3 +/+ mice contained more *L. major* DNA compared to IL-3 -/- mice Interestingly, two mice from the IL-3 -/- group showed C_T values which were only slightly lower than uninfected ear tissue. Such a high CT value indicates low amounts of *L. major* DNA which could be caused by small amounts of viable parasites that persisted at the site of infection or, alternatively, fragments of DNA from dead parasites that remained after parasite clearance (73, 74). This is an interesting finding because it may

suggest in the absence of IL-3, BALB/c mice mount a more protective Th1 response against *Leishmania* infection. However, to verify the type of immune response produced by IL-3 +/+ and IL-3 -/- mice, cytokine levels characteristic of those secreted by Th1 and Th2 cells need to be monitored. Such studies are currently underway in our laboratory. Nevertheless, these data suggest at least some of the IL-3 -/- mice were able to more effectively combat the *L. major* parasites than were IL-3 +/+ mice.

Resistance and susceptibility to *L. major* infection has been repeatedly shown to be dependent on the type of adaptive immune response produced by the infected host. Adaptive immunity, including the development of Th1 and Th2 cells, is typically generated in lymphoid tissues like LNs. Therefore, draining LNs of infected mice were evaluated to determine if IL-3 was affecting the local cellular environment. Because LNs provide a venue for the development of resistant and susceptible immune responses to CL, the differences observed in disease severity among IL-3 +/+ and IL-3 -/- mice could be explained if IL-3 has the ability to influence the types of cells present in draining LNs. If IL-3 +/+ and IL-3 -/- mice have different types of cells within their draining LNs, it is highly likely these cells are producing different types of cytokines, which could in turn influence the type of immune response.

To test this idea auricular LNs that drained the lesion of *L. major*-infected IL-3 +/+ and IL-3 -/- mice were collected at four weeks post-infection and monitored for levels of CD4$^+$ T cells and B cells by flow cytometry. In uninfected IL-3 +/+ and IL-3 -/- mice, the levels of CD4$^+$ T cells and B cells were essentially identical. However, four weeks after *L. major* infection, IL-3 +/+ mice had notably lower percentages of CD4$^+$ T cells and significantly higher percentages of B cells compared to IL-3 -/- mice (Fig. 11). These findings indicate IL-3 can either directly, or indirectly, influence the level of lymphocyte populations in

draining LNs during the course of CL. However, the mechanism by which IL-3 is able to affect lymphocyte populations is unknown.

Increases in B cell percentages at four weeks post infection by IL-3 +/+ mice suggests B cell proliferation was induced by some factor(s) at points earlier in infection. Perhaps the most potent factor which drives antigen-activated B cell proliferation is IL-4. Moreover, IL-4 is the primary cytokine implicated in promoting Th2 immune responses that leads to susceptibility to *Leishmania* infection. Therefore, higher numbers of B cells in IL-3 +/+ mice may indicate there is increased production of IL-4 in infected IL-3 +/+ mice, potentially resulting in a more polarized Th2 immune response. Since expansion of IL-4-producing basophils is acutely dependent on IL-3, it would make sense to observe higher levels of IL-4 in IL-3 +/+ mice compared to IL-3 -/- mice. Ongoing experiments in the laboratory are aimed at assessing IL-4 levels in IL-3 +/+ and IL-3 -/- mice.

In pilot experiments of *L. major* infection, BALB/c IL-3 +/+ mice had increased disease severity compared to similarly infected BALB/c IL-3 -/- mice. IL-3 is particularly influential on basophils; it is the major cytokine that increases the function and proliferation of basophils in response to infection (59). Increasing the numbers and functional ability of basophils influences the amount of secretory products (i.e. IL-4) these cells produce. Therefore, it was hypothesized that increased disease severity seen in *Leishmania*-infected IL-3 +/+ mice is caused by IL-3-stimulated basophils promoting Th2 susceptible immune responses, possibly through their ability to secrete IL-4.

To begin evaluating this hypothesis, it was first determined if *L. major* infection induced the expansion of basophil populations in bone marrow which is where basophil development occurs. At physiological conditions, basophils comprise approximately 0.5 - 0.9 % of bone marrow samples from BALB/c mice (59). Because basophil levels are so low at

homeostasis, relatively small increases to 2 - 4% would represent significant basophil proliferation (59) . To monitor the proliferative ability of basophils in response to *Leishmania* parasites, bone marrow samples were collected from uninfected and *L. major*-infected BALB/c IL-3 +/+ mice and subjected to flow cytometry. IL-3 -/- mice were not initially evaluated in these studies because IL-3 is the only known factor to promote proliferation of these cells, and it was assumed that in the absence of IL-3, there would be no basophil expansion. These studies showed that at five weeks post-infection, basophil levels in bone marrow from *L. major*-infected IL-3 +/+ mice were essentially the same as basophil levels from uninfected IL-3 +/+ mice (Fig. 12). Although no basophil proliferation was detected at five weeks post infection, this does not conclusively show that basophil populations do not expand during the course of CL. In fact, basophil populations in bone marrow could have expanded earlier in response to *L. major* but had returned to baseline levels by 5 weeks post-infection. To ensure *Leishmania* infection does not induce early expansion of basophil populations, ongoing studies are being completed which look at basophil levels in bone marrow at earlier time points post-infection.

Basophil levels were also monitored in draining LNs to determine if these cells migrate to lymphoid tissue in response to *L. major* infection. Typically, basophils are not detectable in LNs. However, recent work describing the immune response to nematode infections reported that basophils transiently migrate to LNs and can influences the type of immune response by their ability to secrete IL-4 (75). To monitor lymphoid tissue for basophil migration, *L. major*-infected IL-3 +/+ mice were sacrificed at days four, nine, and four weeks post-infection to collect LN cells for flow cytometric analysis. However, no IgE^{+}, CD49b^{+} basophils were detected in draining LNs at any of these times following *L. major*

infection (Fig. 13). CD49b$^+$ is surface protein expressed by basophils, and together with IgE staining, is commonly used to distinguish basophils by flow cytometry.

Because basophil were undetectable at the indicated times, these studies suggest that basophils may not migrate to any significant degree to draining LNs of BALB/c mice in response to *Leishmania* infection. However, recent experiments which were able to detect basophils in LNs suggest basophil migration to LNs is transient (75). Thus, if LNs are not evaluated at the optimal time, evidence of basophil migration could be missed entirely. Therefore, it is possible *L. major* infection does induce basophil migration to LNs but, due to the transient nature of this migration, we were unable to identify basophils at the specified time points examined.

Our studies indicate that IL-3 influences the course and outcome of *L. major* infection in susceptible BALB/c mice. It was originally hypothesized that susceptibility to *Leishmania* parasites would be promoted by IL-3 through its ability to increase basophil numbers. However, no increases in basophil proliferation were detected in the bone marrow or draining LNs of   *L. major*-infected IL-3 +/+ mice, suggesting IL-3 may not induce basophil proliferation during CL. Although no increases in basophils were observed, the hypothesis cannot be fully rejected because IL-3 could act by increasing the functional status of basophils independent of their proliferation. For instance, IL-3 stimulation could result in elevated IL-4 production by basophils even though basophils remain at approximately baseline levels. In fact, some studies which indicate that basophils drive Th2 responses have observed that basophil numbers do not increase during these responses (76).

Experiments which monitor basophil levels in different tissues at multiple times post-infection will be needed in order to rule out the possibility that basophils function during CL. For example, monitoring bone marrow samples earlier than five weeks following

infection could rule out the chance that expansion of basophil populations occurs early during infection and returns to baseline levels by five weeks. Also, basophil levels in LNs from infected mice could be examined on a day-to-day basis to ensure a transient migration of these cells to lymphoid tissue is not overlooked. Along with monitoring different time points following infection, tissues which were not evaluated in the current study, such as infected ears and blood, should be monitored for basophil increases. If still no basophil proliferation is noted at these sites and time intervals, the functional ability of these cells should be evaluated during *Leishmania* infection. For example, studies could be initiated to determine if basophils present following *L. major* infection are able to produce more IL-4 than basophils existing under physiological conditions.

If basophil proliferation and/or function are not increased during CL, IL-3 is likely functioning independent of basophils. Although the major functions of IL-3 appears to be its ability to increase the proliferation and secretory ability of basophils, conclusive evidence regarding the involvement of basophils will require the use of animals selectively deficient in basophils. If basophils do not have a role in CL, then IL-3 may influence the course and outcome of Leishmania infection by acting on other cell types known to be influenced by this cytokine.

IL-3 is known to play a major role in the growth and differentiation of rodent mast cells (77). IL-3 has also been shown to upregulate mast cell release of mediators like histamine (78) and anti-inflammatory cytokines such as IL-10 and IL-13 (79). Furthermore, infection of BALB/c mice with *L. major* induces significant mast cell degranulation at early stages of the infection (80), and promotes mast cell proliferation at the site of infection (81). Because IL-3 is known to influence the development and function of mast cells, and because mast

cells have been shown to influence the course of *Leishmania* infection, it is feasible to speculate that the function of IL-3 during CL could be mast cell dependent.

IL-3 could influence the course and outcome of *L. major* infection through its role in the recruitment of effector cells. IL-3 participates in inflammatory responses by inducing the expression of adhesion molecules on endothelial cells (82, 83). The expression of cell adhesion molecules by blood vessel endothelial cells near the site of infection facilitates diapedesis (migration of cells from blood into tissue) of effector cells. In turn, increased diapedesis would cause higher levels of leukocyte migration to the site of infection resulting in an advanced degree of inflammation. Therefore, it is possible that IL-3 functions during CL to promote inflammation through the expression of cell adhesion molecules, which would explain why higher degrees of inflammation were detected in *L. major*-infected IL-3 +/+ mice.

Differences seen between *L. major*-infected IL-3 +/+ and IL-3 -/- mice may also be due to this cytokine's ability to influence macrophages. IL-3 has been shown to modulate the expression of certain molecules on macrophages resulting in different levels of macrophage activation (84). These cells play such a vital role during CL because amastigotes remain primarily in macrophages during *Leishmania* infection. Past studies have monitored the effects of IL-3 on macrophages in the context of *Leishmania* infection. For example, Feng *et al.* (1988) showed addition of IL-3 to cultured macrophages infected with *L. major* promoted the growth of intracellular parasites (42). To further address the role of IL-3 and macrophages, Saha *et al.* (1999) examined leishmanial-antigen pulsed macrophages derived *in vitro* with either IL-3 or GM-CSF that were adoptively transferred into BALB/c mice just prior to infection with *L. major*. While BALB/c mice receiving GM-CSF-derived macrophages significantly resist infection, BALB/c mice receiving IL-3-derived

macrophages exacerbated disease as assessed by footpad thickness (43). Currently, *in vivo* experiments are being conducted to determine the effects of IL-3 on macrophage function during CL. These studies are focused on the activational status of macrophages at the site of *L. major* inoculation to determine if endogenous IL-3 does in fact induce anti-leishmaniacidal effects.

This study has established a novel finding which shows that endogenous IL-3 significantly contributes to the susceptibility of BALB/c mice during *L. major* infection. This is supported by data showing that *L. major*-infected IL-3 +/+ mice have increased inflammatory lesions and increased parasite burdens relative to similarly infected IL-3 -/- mice. That IL-3 may function in CL to promote a Th2-dominated immune response is supported by our data that shows infected IL-3 +/+ mice have increased levels of B cells and decreased of CD4$^+$ T cells. Although these studies indicate that basophil numbers do not increase during infection at the time points examined, these data do not rule out the possibility that physiological levels of IL-3-dependent basophils influence the immune response. Because IL-3 can influence cellular differentiation, and expression of molecules by a number of different cell lineages, several possibilities could explain how IL-3 functions during CL. It is unlikely that the function of IL-3 during this disease can be completely explained by its effects on a single population of cells. Instead the differences observed in IL-3 +/+ and IL-3 -/- are likely caused by IL-3 acting on numerous cell types in a concerted fashion. Although the mechanism(s) by which endogenous IL-3 functions during CL has yet to be fully determined, the current study has established an important and novel finding regarding the immune response to CL. Furthermore, the current work justifies further examination of the role of IL-3 in CL and will reveal new insights on the role of IL-3 in health and disease.

Appendix

The following figures have been added for further clarification and are intended to supplement both the materials and methods and the results sections.

Figure 14 Genotyping IL-3 +/+ and IL-3 -/- mice. Constructs used to produce IL-3 -/- mice allow the genotypes to be distinguished by PCR. Using specific primers permits the amplification of DNA fragments which are different sizes and correspond to either IL-3 +/+ (1200bp) or IL-3 -/- (800bp) mouse DNA. Tail biopsies were collected from both groups of mice and DNA was extracted by enzyme degradation and subjected to PCR. After PCR, DNA products were then run on an agarose gel and stained with ethidium bromide to detect amplicons.
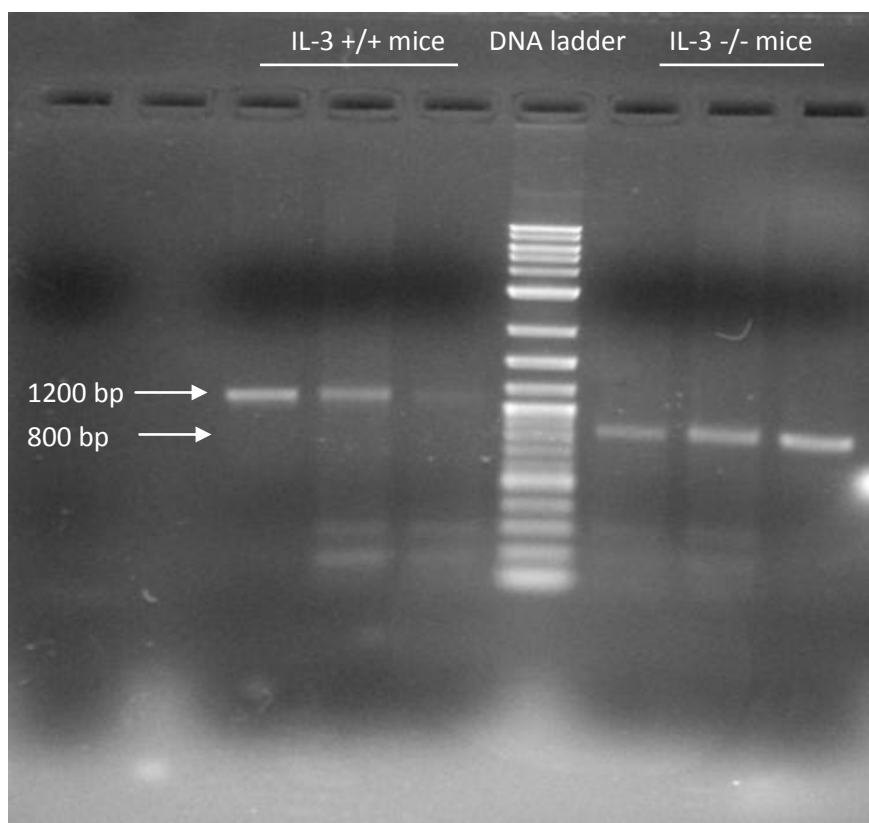
Figure 15 Images of *L. major* parasites in culture at different developmental stages. Cytospins of *L. major* culture samples were fixed and stained with Giemsa for 15 min. Plates A, B, and C represent images of parasites taken at days 2, 3, and 4, respectively. Plate D represents purified metacyclic promastigotes isolated from culture on day 5 by negative selection via peanut agglutinin. Metacyclic parasites display a homogeneous morphology and can be distinguished from less mature promastigotes based on their slender cell body, long flagellum, and tapered anterior tip. In each plate, scale bars represent 10 μm.
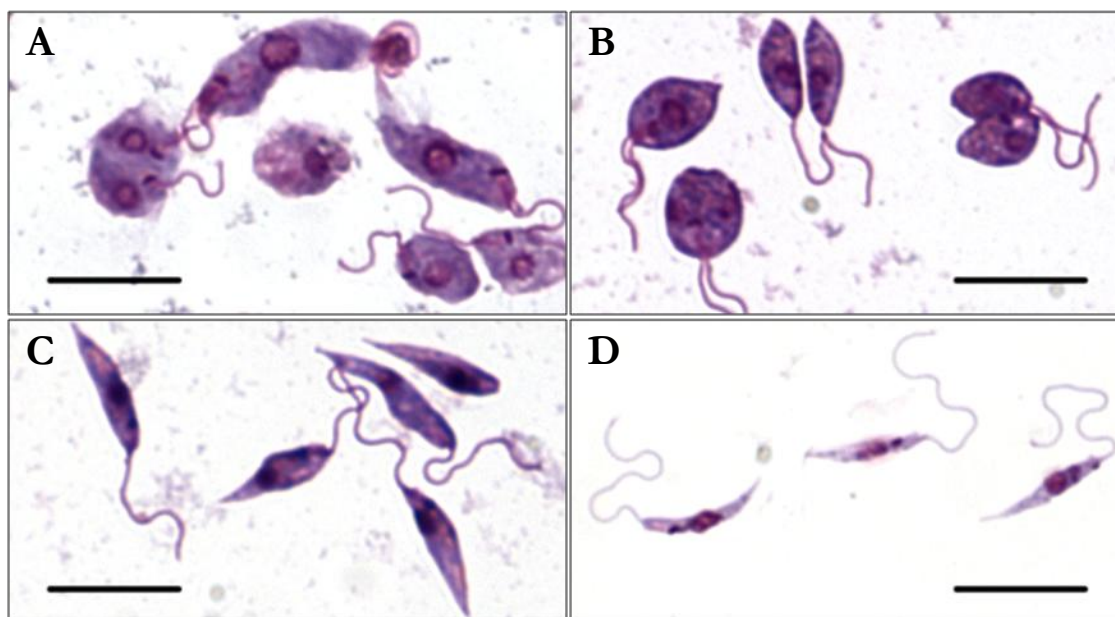
Figure 16 *Leishmania major* growth curve. *L. major* parasites were cultured in c-M199 media for 5 days to facilitate the development of infective stage metacyclic promastigotes. Metacyclics were then separated from the rest of the culture to initiate infections via negative selection with PNA. Data represents total number of parasites (left y axis) each day after initiating the culture, and the total number of metacyclic promastigotes (right y axis) isolated on day 5 of culture.
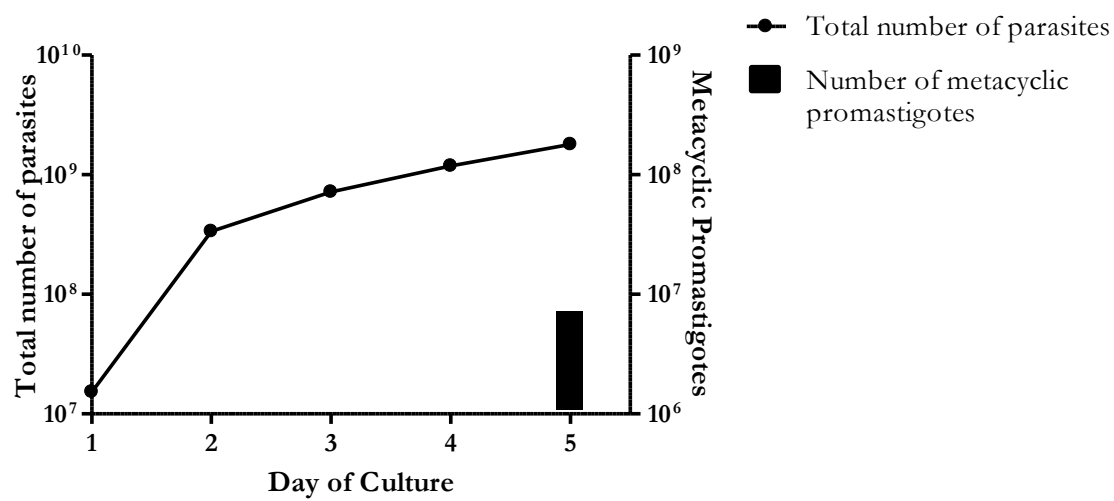
Figure 17 *L. major* metacyclic promastigotes being injected into the ear dermis of an anesthetized mouse.

Figure 18 Image of *L. major*-infected and PBS-injected mouse ears. (A) PBS-injected mouse ear showing no pathology or visually detectable inflammation. (B) *L. major*-infected ear showing distinct lesion and inflammation at site of infection.
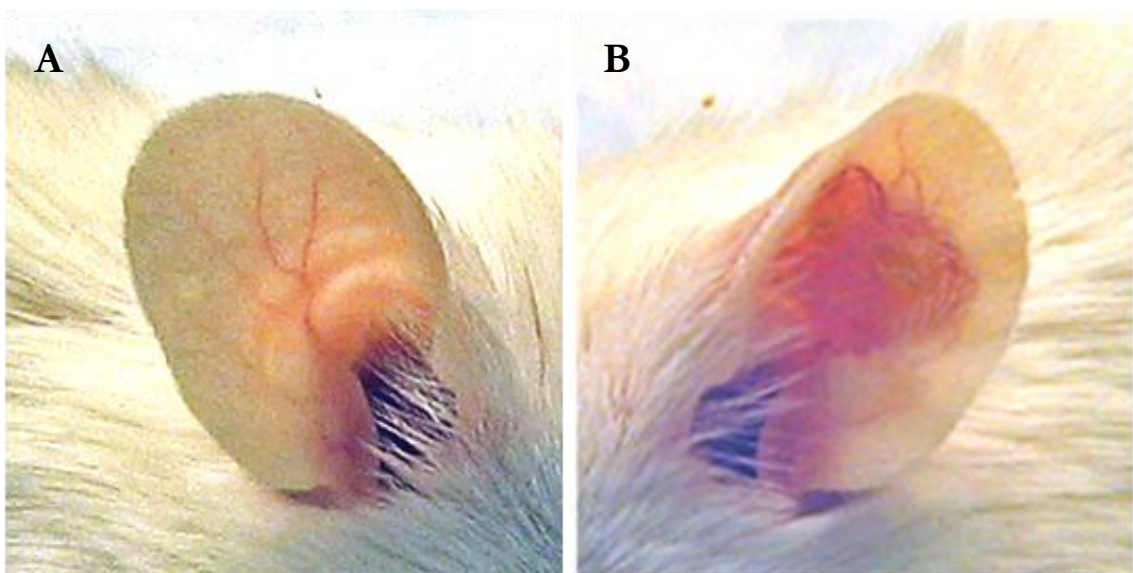
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