The Role Of Phosphoinositide 3-Kinase Gamma In The Host Immune Response Against Cutaneous Leishmaniasis Caused by *Leishmania mexicana*

DISSERTATION

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ABSTRACT

The leishmaniasis are a group of diseases resulting from infection with protozoan parasites of the genus *Leishmania*. Cutaneous leishmaniasis, caused by species such as *Leishmania mexicana*, is the most widespread form of disease and is common throughout Central and South America, Africa, India, and Southwest Asia. Cutaneous infection results in the formation of well defined, localized lesions that generally self-resolve without need for treatment. However, some patients experience progressive disease and develop non-healing chronic infections. Problems associated with enhanced drug resistance by parasites and increased patient-unresponsiveness towards standard chemotherapeutics has become a significant concern for health officials worldwide. With 350 million people currently living in areas of active parasite transmission, the need for effective therapeutics to treat infection is considered a primary goal of The World Health Organization and health officials worldwide.

The Phosphoinositide 3-kinases (PI3Ks) are a large family of evolutionarily conserved protein kinases involved in intracellular signal transduction. PI3Ks are grouped into three classes and include Class IA and IB, Class II, and Class III PI3Ks. Studies using broad-spectrum PI3K inhibitors such as wortmannin and LY294002 have
shown that Class I PI3Ks are involved in phagocytosis and in mediating entry of parasites such as *Trypanosoma cruzi* into host cells however, the precise role of each isoform remains unclear. Class IB PI3Kγ is expressed primarily by immune cells and has been shown to play a critical role in chemo-attractant induced cell migration by controlling actin cytoskeletal reorganization.

In this document, we have characterized a role for PI3Kγ in cutaneous leishmaniasis caused by *Leishmania mexicana*. Using a PI3Kγ-selective inhibitor, AS-605240, and PI3Kγ-deficient mice, we demonstrate that PI3Kγ contributes to the pathogenesis of cutaneous leishmaniasis by mediating recruitment of phagocytes and regulatory T cells to the site of infection and by facilitating parasite entry into phagocytes. Furthermore, we demonstrate that AS-605240 is as effective as standard anti-leishmanial chemotherapy with sodium stibogluconate in limiting parasite growth after infection. These findings reveal a novel role for PI3Kγ in *Leishmania mexicana* invasion and establishment of chronic infection, and demonstrate that therapeutic targeting of host pathways involved in establishment of infection is a viable treatment strategy for treating cutaneous leishmaniasis caused by *Leishmania mexicana* and possibly other intracellular pathogens of phagocytes.
Dedication

To My Family and To My Friends
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1.1 SUMMARY:

The diseases termed “leishmaniasis” were first described in 1903 by William Leishman and Charles Donovan (1). Leishmaniasis is a zoonotic disease resulting from infection with protozoan parasites of the genus *Leishmania*, of which approximately 20 different species pathogenic for humans have been described (1-4). Experimental models of murine leishmaniasis have provided an excellent tool for investigating the mechanisms involved in host defense against intracellular pathogens and for understanding the complexities of immune regulation as a whole. The PI3-kinases are a large family of evolutionarily conserved lipid kinases that play important roles in numerous cellular functions. PI3Kδ and PI3Kγ are two Class I PI3K isoforms preferentially expressed by cells of the immune system and are known to regulate various stages and components of the host inflammatory response. For this reason, much interest has been focused on the design and development of PI3K isoform-selective pharmaceutical inhibitors for use as treatment against numerous inflammatory diseases. In this document I discuss significant findings characterizing a role for PI3Kγ in mediating host susceptibility to cutaneous leishmaniasis obtained from a mouse model of cutaneous *Leishmania mexicana* infection.
and provide evidence to suggest that therapeutic targeting of PI3Kγ using isoform-selective PI3Kγ inhibitors is a viable strategy for treating cutaneous.

1.2 PARASITES, LIFE CYCLE, AND DISTINCTION BETWEEN DISEASES:

Leishmaniasis is a vector-borne disease transmitted by *Phlebotomus* and *Lutzomyia* sand flies in the Old and New World, respectively (1, 3, 5). Natural transmission occurs when an infected sand fly takes a blood meal and deposits highly infective, metacyclic promastigotes into the dermis of susceptible mammalian hosts (6). *Leishmania* spp. are obligate intracellular pathogens in mammals and parasite survival and replication requires that parasites be rapidly internalized into host cells. Promastigotes are phagocytosed by neutrophils and macrophages with the latter acting as the definitive host cell for parasite survival and replication within the mammalian host. Inside macrophages, promastigotes transform into smaller, rounded, aflagellated amastigotes, which undergo unrestricted replication within the phagolysosome (4, 6, 7). Lysis of infected macrophages releases infectious amastigotes, which target uninfected macrophages recruited to the site to potentiate infection. The life cycle is continued when an uninfected sand fly takes a blood meal and ingests either free amastigotes in the blood or cells harboring intracellular amastigotes. Inside the sand fly mid-gut, amastigotes transform back into infectious promastigotes (6, 8).

The leishmaniasis comprises a collection of disease syndromes, which differ in their clinical, histological, and immunological features/characteristics. The clinical manifestations of disease differ depending on causative species and occur in three major forms: cutaneous, mucosal, and visceral leishmaniasis (1-4, 7, 9).
CUTANEOUS LEISHMANIASIS:

Cutaneous Leishmaniasis (CL), also known as “Chiclero’s Ulcer” and “Oriental Sore” in the New and Old World, respectively, is the most prevalent form of disease and is estimated to affect between 1.5-2 million people worldwide (1, 3, 4). New World CL is most commonly caused by the species *Leishmania mexicana*, *Leishmania braziliensis*, *Leishmania amazonensis*, *Leishmania panamensis*, and *Leishmania guyanensis*, whereas *Leishmania major* and *Leishmania tropica* are primarily responsible for CL in the Old World (3, 4, 9). Cutaneous leishmaniasis is characterized by the development of localized, ulcerating lesions, which typically appear on areas of the body exposed to sand fly bites, such as the face, arms, and legs (3, 4, 8). Cutaneous infections are most often self-limiting and resolve within 2-18 months without need for treatment (4, 10), however reports of patient unresponsiveness towards treatment and the emergence of drug resistant strains is becoming an increasing concern for health officials worldwide (11-13).

MUCOCUTANEOUS (MUCOSAL) LEISHMANIASIS:

Mucosal Leishmaniasis (ML) is characterized by the spread of parasites to infect the tissues of the nasal and oral mucosa and is potentially life threatening if left untreated (14). ML is associated with infection by *Leishmania* parasites of the subgenus *Viannia*, such as *Leishmania (V.) braziliensis*, *Leishmania (V.) panamensis*, and *Leishmania (V.) guyanensis* (4, 14, 15) in the New World. Mucosal leishmaniasis typically occurs in two phases, the first being the development of a cutaneous lesion following the bite of an
infected sand fly followed by secondary involvement of mucosal tissues (14, 16). Mucosal infection is relatively rare occurring in only 1-10% of infected patients, and is typically associated with hyperactive immune responses leading to uncontrolled inflammation and tissue destruction despite a low number of parasites typically present within the lesions (17, 18).

**VISCERAL LEISHMANIASIS:**

Visceral Leishmaniasis (VL) is the most severe form of disease, resulting in death of infected patients if left untreated. In the visceral form of infection, parasites disseminate throughout the mononuclear phagocyte system to infect the spleen, liver, and bone marrow (1,19). Patients with visceral disease suffer from fever, general cachexia, and severe hepatosplenomegaly resulting from heavy parasite burdens within the infected organs (1, 7). VL is associated with species belonging to the *Leishmania donovani* complex including *Leishmania donovani*, *Leishmania infantum*, and *Leishmania chagasi* (20). An estimated 500,000 new cases of VL are reported each year and the World Health Organization suggests that VL is responsible for nearly 50,000 deaths annually.

A fourth manifestation of disease is Post-kala-azar dermal leishmaniasis (PKDL), which occurs in anywhere from 5-10% to 50-60% of patients in India, Sudan, and the surrounding areas (19, 20) PKDL is observed in patients with a previous history of visceral disease who resolved infection and appeared seemingly well (19, 20). PKDL presents as dermal lesions, which appear on the face and spread to the torso, arms, legs, neck, and back (19, 20). While patients with VL are typically unwell and harbor
detectable parasite burdens within their lymph nodes, spleen, and bone marrow, PKDL patients are generally well, with parasites only detectable within the skin (19-22). These lesions harbor viable parasites and therefore PKDL is considered a reservoir for *Leishmania* parasites.

Species associated with PKDL are those associated with VL although PKDL is almost exclusively observed in patients infected with *Leishmania donovani* (19-22).

1.3 EPIDEMIOLOGY, CURRENT THERAPY, AND VACCINES:

Leishmaniasis is endemic in 88 countries and affects nearly 12 million people worldwide. The WHO estimates that an additional 350 million people currently live in areas of active parasite transmission (1, 2, 4, 9). Cutaneous leishmaniasis (CL) affects 1.5-2 million people each year and is endemic throughout Central and South America, Africa, and the Middle East with over 90% of cases occurring in Afghanistan, Pakistan, Syria, Saudi Arabia, Algeria, Iran, Brazil, and Peru (4, 9). Mucocutaneous leishmaniasis (ML) occurs in only 1-10% of patients suffering from cutaneous leishmaniasis in the New World (14-18). Visceral leishmaniasis (VL) is the second largest killer among infectious parasitic diseases and is responsible for more than 50,000 deaths each year (3, 4, 23). 500,000 new cases of visceral disease are reported each year with 90% of cases occurring in Bangladesh, India, Nepal, Sudan, Brazil, and Peru (1, 2).
CURRENT THERAPY:

Pentavalent antimonials sodium stibogluconate (Pentostam®) and meglumine antimoniate (Glucantime®) have been used clinically for over 60 years and remain the first-line treatment for all forms of leishmaniasis in most endemic regions (1, 13, 24-29). Standard therapy typically requires daily injections (i.m. or i.v.) of antimony (20 mg/kg) for a period of 20-30 days and is associated with severe adverse side effects including fatigue, body ache, cardiac and hepatic toxicity, pancreatitis, and pneumonitis (1, 25, 30, 31). Due to the adverse effects, prolonged treatment periods, high costs, and poor distribution of the drug, antimony therapy is regarded as unfavorable and is often met with poor patient compliance. Furthermore, an increase in the emergence of drug resistance towards antimony therapy has led to the search for novel therapeutics to treat more resistant forms of disease (24, 30, 31).

Second-line therapy is typically treatment with aromatic diamidines Amphoceritin B or Pentamidine (1, 24-26). Amphoceritin B, an anti-fungal compound, induces parasite killing by interacting with sterols to disrupt the integrity of the cell membrane and is typically administered at a dosage of 1 mg/kg on alternate days for a period of 30 days (25, 32, 33). Pentamidine exerts its leishmanicidal effects through lethal interactions with parasite DNA within the kinetoplast, ultimately leading to destruction of the mitochondrion (26, 34). As with pentavalent antimony, the use of Amphoceritin B and Pentamidine therapy is limited by severe renal toxicity, cardiac toxicity and adverse side effects including rigor, fever, and chills (25, 26, 32, 35, 36). Furthermore, use of Pentamidine isothionate has been associated with the development of irreversible
diabetes mellitus (36, 37). A modified liposomal Amphoceritin B treatment (Ambisome®) has been developed that is highly effective and requires shorter treatment periods, however high drug costs have limited the use of this therapy in developing countries where the diseases are endemic (10, 37-40).

The oral drug miltefosine is among the latest drugs to be introduced clinically and has demonstrated a high level of efficacy in the treatment of visceral leishmaniasis (26, 31, 37, 38, 40-43). Miltefosine is an alkylphosphocholine analogue and functions to interfere with cell signaling and membrane biosynthesis, however the precise mechanisms by which miltefosine exerts its anti-leishmanial effects remains unclear (38, 44-46). Miltefosine has been shown to induce apoptotic-like death of Leishmania parasites by several mechanisms, including interference of key steps involved in the biosynthesis of membrane lipids and glycoproteins, the elongation of fatty acids, disruption of mitochondrial function, and inhibition of choline transport (44, 47-54). Oral treatment with miltefosine is largely effective in the treatment of VL when administered at a dosage of 100-150 mg/day for a period of 20-28 days and is associated with limit adverse side effects, which are typically transient and non-life threatening, the most common being gastrointestinal upset (40, 55-57) The use of oral miltefosine to treat cutaneous leishmaniasis has been demonstrated to have varying degrees of efficacy depending upon both the causative species and endemic region, and is not considered as effective for treatment of CL as standard is standard chemotherapy with antimonials (39, 58-60).
ANTI-LEISHMANIA VACCINES:

To date there are no effective vaccines available for human use for protection against any parasitic diseases, including leishmaniasis (61). Patients that successfully resolve *Leishmania* infection however, acquire life-long protective immunity to re-infection, suggesting that the development of successful vaccines is feasible. ‘Leishmanisation’, or inoculation of individuals with live, virulent *Leishmania* parasites, has been used for over a century to induce protective immunity against re-infection (62-65), however this method has been largely abandoned due to obvious safety concerns and issues of standardization (11, 62, 66).

The ‘ideal’ vaccine should provide broad-spectrum protection against all pathogenic species of *Leishmania*. First generation anti-*Leishmania* vaccines using killed parasites have largely replaced ‘Leishmanisation’ but have failed to show promising results in clinical trials (61, 67, 68). Second-generation vaccines composed of genetically modified parasites, recombinant proteins, peptides, and DNA vaccines are under development but promising findings in the laboratory using mouse models have proven difficult to translate in the field to human disease (11, 61). Leish-111f, a peptide-based vaccine is the only second-generation vaccine to reach clinical trials (61, 66, 69, 70). Leish-111f, composed of three fused *Leishmania* spp. proteins into a single polyprotein, has shown protective efficacy against infection in numerous experimental murine models and has completed Human Phase I and II clinical trials in Brazil, Peru, and Columbia, as well as Phase I trials in India (61, 66, 69, 70, [www.clinicaltrials.gov](http://www.clinicaltrials.gov)).
The development of effective vaccines against leishmaniasis has been slow, with large pharmaceutical companies seeing little incentive to invest in the research and development required for production of an effective and safe vaccine for public use. However with 350 million people estimated to live in areas of active parasite transmission and increased reports of drug resistance, The World Health Organization has recognized leishmaniasis as a global health priority and is providing funding with the goal of complete disease eradication by the year 2015 (25, 71).

1.4 INNATE IMMUNITY AND LEISHMANIA INFECTION:

THE SKIN:

The skin plays an important role in innate immunity and protection against Leishmania spp. infection by acting as a physical barrier to the entry parasites and by initiating the infiltration of inflammatory cells necessary to control infection (72). Epithelial cells express Toll-like receptors (TLRs), pattern recognition receptors that function to detect invading pathogens (72-74). TLR signaling by epithelial cells induces the production of antimicrobial peptides, cytokines and chemokines important for innate immunity against microbial pathogens (72, 73). Keratinocytes represent the major cell population within the epidermis and in humans, have been demonstrated to express functional TLRs (72-75). Differential TLR activation has been associated with the production of the pro-inflammatory cytokine tumor necrosis factor-α (TNF-α), the neutrophil chemotactic factor IL-8 (CXCL8), the monocyte and basophil chemokine CCL2, macrophage inflammatory protein-3 (CCL20), as well as the production of
chemokines CCL27, CXCL9 and CXCL10 which are associated with recruitment and activation of memory T cells into the skin and the generation of cell-mediated T-helper type 1 immune responses, as well as to activate the expression of inducible nitric oxide synthetase (iNOS) (73-75).

**NEUTROPHILS:**

*Leishmania* parasites evade the barrier function of the skin by gaining access to the host using breakages in the skin caused by the bite of the sand fly during feeding. Neutrophils, which rapidly infiltrate the skin in response to tissue damage, microbial products, and chemokines, play a critical role in the wound-healing response to tissue damage and in early innate immune responses to *Leishmania* infection (76-80). Neutrophils are present in the circulating blood and are rapidly recruited into the tissue in response to tissue damage and/or microbial products (76-78). IL-8, a CXC chemokine (CXCL8) primarily secreted by epithelial cells, keratinocytes, fibroblasts, endothelial cells, as well as by neutrophils themselves, is a potent neutrophil chemo-attractant and functions to recruit neutrophils to the site of infection following a sand fly bite (76). Human neutrophils were shown to produce IL-8 in response to *Leishmania* infection, and transcription of KC, a functional homologue of IL-8 expressed in mice, was also found to be up-regulated in the skin of *Leishmania*-infected animals *in vitro* (78, 81, 82).

*Leishmania* promastigotes have also been shown to secrete Leishmania Chemotactic Factor (LCF). LCF has been shown to function as a potent neutrophil chemo-attractant by binding to fMLP receptors on the cell surface (81).
The vast majority of research focused on early *Leishmania*-neutrophil interactions has been conducted using mouse strains which are genetically susceptible (BALB/c) or resistant (C57BL/6) to infection with *Leishmania major*, the causative agent of cutaneous leishmaniasis in The Old World. Neutrophils, which enter the tissues from the blood, form a ‘plug’ at the site of tissue damage and rapidly phagocytose promastigotes deposited in the skin (77). *In vivo* analysis of infected tissues has demonstrated that neutrophils rapidly accumulate at the site of infection and harbor viable parasites within phagolysosomes to promote the early establishment of infection and facilitate establishment of chronic infection by protecting parasites from extracellular destruction (77). Neutrophils have also been suggested to act as a ‘‘Trojan Horse’’ in establishing *Leishmania* infection by facilitating parasite entry into macrophages as they phagocytose parasitized and/or apoptotic granulocytes, mechanisms that fail to induce anti-leishmanial host responses (83-85). While there is clear evidence that neutrophils play an important role in innate immune responses during the early stages of *Leishmania* spp. infection, the precise role of these cells in promoting disease exacerbation or protection against infection has revealed controversial results, as both protective and non-protective roles for these cells have been described (77, 80, 85-89).

**MACROPHAGES:**

Macrophages are the definitive host cell targeted by *Leishmania* parasites and play an important role as both the effector cell required for parasite killing and control of *Leishmania* infection as well as function as a reservoir for parasite survival and
replication within the mammalian host (96). Early entry into host cells is a receptor-mediated process and is largely facilitated by promastigote binding to CR1/CR3 on macrophages (96, 99). Entry into macrophages via CR3 is considered a ‘silent’ mechanism of entry into host cells, as uptake via CR3 fails to illicit the production of reactive oxygen species and antimicrobial responses capable of killing intracellular parasites (97, 98, 100, 101). Within macrophages, *Leishmania* promastigotes transform into rounded, aflagellated amastigotes, which are resistant to the low pH and hydrolytic components within the phagolysosome. Amastigotes survive and replicate within the phagolysosome of macrophages, ultimately leading to cell lysis and the release of infectious amastigotes to infect phagocytes recruited to the site of infection.

Control of *Leishmania* infection is critically dependent upon the activation of macrophages to produce nitric oxide (NO). Macrophages derived from mice genetically deficient of NOS2 (inducible nitric oxide synthetase) are unable to eliminate parasites *in vitro* and NOS2-deficient animals fail to control *Leishmania* infection (96, 102). *Leishmania* parasites have been shown to inhibit NO production by infected macrophages in response to stimulation with LPS and/or IFN-γ, thus promoting parasite survival within the host (96, 103, 104). *Leishmania*-infected macrophages also show altered MHC II expression and impaired antigen presentation and are impaired in their ability to produce numerous cytokines involved in the generation of a protective, pro-inflammatory immune response such as IL-1, TNF-α, and IL-12 (96, 105-108). In addition to inhibiting the production of protective, pro-inflammatory cytokines, *Leishmania* parasites promote the production of IL-10, a potent immunosuppressive cytokine associated with susceptibility
to infection and disease progression, by infected macrophages *in vitro* (96, 109). The significance of IL-10 as a susceptibility factor in *Leishmania* infection *in vivo* is evidenced by the inability of transgenic mice over-expressing IL-10 to control *Leishmania* infection (96, 110).

**DENDRITIC CELLS:**

Dermal dendritic cells (dDCs) and Langerhans cells are the major sentinel professional antigen-presenting cell (APC) present within the skin. dDCs play an important role as a link between innate and adaptive immune responses against *Leishmania* infection by facilitating the transport of antigen within the skin and draining lymph nodes (111, 112). Dendritic cells are capable of activating naïve T cells in the lymph nodes and therefore play an important role in linking both innate and adaptive immune responses against *Leishmania* infection (113, 114). DCs and Langerhans cells exist in the dermis in an immature state, optimized for the detection, phagocytosis, and processing of microbial antigens that breach the epithelial barrier of the skin (113, 114). DCs have been shown to interact with *Leishmania* parasites via pattern recognition receptors such as TLR2, which typically induces signaling leading the activation of these cells, increased expression of costimulatory molecules (CD40, CD80, CD86), and enhanced antigen presentation via MHCII to CD4+ helper T cells (114, 115). Activated DCs are an important source of the pro-inflammatory cytokine IL-12, a Th1-associated cytokine required for activation of Th1-type CD4+ T cells and the development of
effective cell-mediated immune responses capable of controlling parasite growth and replication within the host (114).

**NATURAL KILLER (NK) CELLS:**

Natural killer (NK) cells play an important role in the host immune response against intracellular pathogens by their ability to lyse virally infected and/or tumor cells as well as by secreting cytokines that can promote the development of protective Th1-type T cell responses (116-118). Several studies have demonstrated that NK cells contribute to protective immune responses in experimental models of cutaneous *Leishmania major* infection in mice (119, 120). Antibody-mediated depletion of NK cells in resistant C57BL/6 was found to promote disease exacerbation in *Leishmania major*-infected animals and was associated with significant tissue inflammation and higher parasite burdens at the site of infection as compared to untreated animals. Similarly, NK activation by poly I: C was shown to reduce parasite burdens and increase early resistance against *Leishmania major* infection in genetically susceptible BALB/c mice (120). While NK cells are likely not essential for protection against *Leishmania major* infection, IFN-γ production by NK cells likely contributes to early protection against *Leishmania* infection by promoting the expansion and differentiation of CD4+ helper T cells into Th1-type subsets required for controlling parasite replication and resolution of disease (119-122).
1.5 ADAPTIVE IMMUNITY AND LEISHMANIA INFECTION:

Control of *Leishmania* infection is critically dependent upon the development of cell-mediated immune responses capable of activating macrophages to kill intracellular parasites. Experimental models of cutaneous *Leishmania major* infection in mice provided the first evidence for the relevance of Th1/Th2 balance in mediating disease outcome *in vivo* and were based largely on studies using mouse strains genetically susceptible (BALB/c) or resistant (C57BL/6) to *Leishmania major* infection (123).

The genetic background of the host plays an important role in determining disease outcome (123, 124). While C57BL/6 mice exhibit a resistant phenotype and control *Leishmania major* infection, BALB/c mice fail to control parasite replication, exhibit systemic parasite dissemination and ultimately succumb to *Leishmania major* infection. The healing- vs. non-healing phenotype exhibited by C57BL/6 and BALB/c mice, respectively, is largely a result of differences in the nature of the helper T cell response generated, with healing being dependent upon the development of cell-mediated Th1-type responses and non-healing, progressive disease resulting from the expansion and activation of Th2-type CD4+ T cells (123).

The resistant phenotype of C57BL/6 mice to *Leishmania major* infection is associated with the development of a protective IL-12-driven Th1-type response and the activation of *Leishmania*-specific CD4+ helper T cells capable of producing IFN-γ (123). The importance of IL-12 in promoting Th1-differentiation and protection against *Leishmania* infection was demonstrated using mice genetically deficient of IL-12. In the absence of IL-12, *Leishmania major*-infected genetically resistant 129/Sv/Ev mice
develop large progressive lesions. In contrast to their wild-type counterparts, IL-12-deficient 129/Sv/Ev mice, like susceptible BALB/c mice, mount a robust Th2-type immune response characterized by high IL-4 and low IFN-γ production by activated CD4+ T cells (124). Further support for IL-12 in promoting the development of Th1-mediated immune responses and protection against infection is evidenced by the ability of exogenous IL-12 treatment to redirect early Th2 responses and promote resistance to *Leishmania major*-infection in susceptible BALB/c mice (125, 126) as well as by studies demonstrating the detrimental effect of IL-12 depletion by anti-IL-12 antibodies on disease progression in resistant mice (126, 127).

The production of IFN-γ by activated *Leishmania*-specific CD4+ T cells is critical for control of *Leishmania* infection as well as infections caused by several other protozoan parasites including *Toxoplasma gondii*, *Plasmodium* spp., and *Trypanosoma cruzi* (128-131). IFN-γ is a potent pro-inflammatory cytokine known to have a multitude of immunomodulatory effects on immune responses both *in vitro* and *in vivo* including up-regulation of MHC expression and the induction of antimicrobial effector molecules and enhancement of microbicidal activity by macrophages and other phagocytes. The hosts’ ability to control *Leishmania* infection is critically dependent upon the activation of macrophages to kill intracellular parasites via the production of NO. IFN-γ produced by activated CD4+ and CD8+ T cells, as well as by activated NK cells, induces NOS2 expression and NO production by macrophages to promote parasite clearance and resolution of infection. The significance of IFN-γ in mediating protective immune responses during *Leishmania* infection was demonstrated in several studies, where
administration of neutralizing anti-IFN-γ antibodies or infection of IFN-γ receptor-deficient mice resulted in increased susceptibility of genetically-resistant mouse strains to infection with *Leishmania major* (132-134). These studies, as well as others, have clearly demonstrated the importance of IFN-γ in mediating microbial killing and promoting the development of protective Th1-type immune responses required for host defense against infection.

In contrast, susceptibility to infection is associated with the development of a non-protective IL-4-driven Th2-type immune response and the production of immunosuppressive cytokines, particularly IL-4 and IL-10, by activated CD4+ T cells (123). A non-protective role for IL-4 in mediating susceptibility to *Leishmania major* infection was initially demonstrated in studies showing that treatment of susceptible BALB/c mice with anti-IL-4 monoclonal antibodies, or infection of IL-4-deficient BALB/c mice, resulted in disease resolution in these mice (135-137). Recent studies have suggested that the biological effects of IL-4 may be more complex than originally thought (123). However, IL-4 production by activated CD4+ T cells is known to actively suppress IL-12-mediated Th1 development and inhibit the production of reactive nitrogen intermediates required for effective parasite killing by IFN-γ activated macrophages thus contributing to host susceptibility.

IL-10 is an anti-inflammatory cytokine produced by many cell types, and like IFN-γ, exhibits numerous biological effects on immune cells. IL-10 has been shown to prevent Th1-development by inhibiting the expression of MHC molecules and the production of pro-inflammatory cytokines such as TNF-α, IL-12, and IL-18 by
macrophages as well as to suppress NO production by activated macrophages to promote parasite survival within the host.

IL-10 has been clearly demonstrated to play a non-protective role in host defense against *Leishmania* infection. In experimental models of *Leishmania major* infection, IL-10-deficiency was found to render genetically susceptible BALB/c mice resistant, as evidenced by the development of smaller lesions with lower parasite burdens in IL-10-deficient animals as compared to their IL-10\(^+/+\) wild-type counterparts (138). Treatment of macrophages with recombinant IL-10 or supernatants from infected-macrophages was also shown to inhibit parasite killing by macrophages in response to stimulation with IFN-\(\gamma\) *in vitro* (138). IL-10 has been demonstrated to play a similar, non-protective role in experimental models of cutaneous *Leishmania mexicana* infection. IL-10-deficiency was found to promote parasite clearance and disease resolution in C57BL/6 mice and was associated with increased expression of IFN-\(\gamma\) and NO production by draining lymph node cells in response to antigen-stimulation *in vitro* (139). Similarly IL-10 production in humans has been shown to correlate with progressive disease in cutaneous *Leishmania major* infection as well as in visceral infection with *Leishmania donovani* (140, 141).

**REGULATORY T CELLS IN LEISHMANIA INFECTION:**

The development of life long immunity to re-infection is a hallmark of *Leishmania* infection and results from a persistence of low numbers of parasites (10\(^2\)-10\(^4\)) within the skin and draining lymph nodes that are sufficient to maintain antigen-specific T cells for rapid protection against secondary challenge. Although parasite persistence is
required for protective immunity against re-infection, the maintenance of viable parasites within the host allows the potential for re-activation of latent infections in immuno-compromised individuals.

In resistant C57BL/6 mice, persistence of Leishmania major parasites within the skin after healing was shown to be controlled by a subset of CD4+CD25+ regulatory T cells (Tregs) by both IL-10-dependent and –independent mechanisms (142). IL-10-production by CD4+ CD25+ regulatory T cells has also been associated with the failure of Th1-promoting vaccines to promote resistance against Leishmania major in susceptible BALB/c mice (143). Similarly, depletion of regulatory T cells renders BALB/c mice resistant to Leishmania major, supporting the notion that regulatory T cells contribute to non-protective immune responses against cutaneous Leishmania infection.

Although IL-10 is required for chronic Leishmania mexicana infection in C57BL/6 mice, regulatory T cells do not appear to play a major role in mediating susceptibility to cutaneous Leishmania mexicana infection as Treg-depletion was found to have no effect on disease outcome in susceptible C57BL/6 mice (144). These findings demonstrate that additional cell types, such as macrophages and conventional CD4+ effector T cells contribute to the production of IL-10 to mediate susceptibility to infection and highlight the differential mechanisms of immune regulation involved in host defense against infection with distinct Leishmania spp.
B CELLS AND ANTIBODIES IN LEISHMANIASIS:

Although some studies have suggested a protective role for B cells host defense against *Leishmania* infection, the activation of B cells and the production of *Leishmania*-specific antibodies are generally associated with non-protective immune responses and disease progression in CL (144-146). IL-4 produced by activated Th2-type CD4+ T cells induces the production of *Leishmania*-specific antibodies by activated B cells that bind to the surface of the parasite to activate complement and opsonization for uptake via CR3/CRI (IgM) or facilitate parasite binding and internalization via FcγRs (IgG). *Leishmania*-bound IgG has been shown to induce IL-10 production by macrophages in response to FcγR ligation and to promote susceptibility to CL infections (138, 139, 146). Mice genetically deficient in β2 microglobulin fail to produce *Leishmania*-specific IgG and are subsequently protected against *Leishmania mexicana* infection (139). These mice are able to resolve lesions and exhibit reduced parasite burdens as compared to wild type mice. These findings are consistent with others describing a detrimental role for B cells and the production of parasite-specific IgG in IL-10-induced susceptibility to *Leishmania* infection.

1.6 PHOSPHOINOSITIDE 3-KINASES (PI3Ks) AND IMMUNITY:

The Phosphoinositide 3-Kinases (PI3Ks) are a large family of evolutionarily conserved lipid kinases involved in intracellular signal transduction (147, 148). The PI3Ks are grouped into three classes based on sequence and structure homology, regulation, and substrate specificity and include Class IA and IB, Class II, and Class III
PI3Ks (147, 148). All PI3Ks function to phosphorylate the 3’-hydroxyl position of inositol-containing phospholipids in the membrane and these 3’-phosphoinositide lipid products play an important role in signal transduction by regulating the localization and activation of various downstream effectors molecules (147, 148). While the role of Class II and Class III PI3Ks remains largely unknown, the vast majority of research has focused on Class I PI3Ks and has revealed an important function for these kinases in mediating signal transduction events involved in various stages and components of inflammatory responses.

Class I PI3Ks are heterodimeric complexes composed of a 110-kDa catalytic subunit (p110α, p110β, p110δ, p110γ) that associates tightly with a regulatory subunit which functions to regulate both the localization and activity within the cell (147, 148). Class I PI3Ks play an important role in intracellular signaling as the only enzymes capable of converting phosphoinositide (4,5) bisphosphate (PtdIns(4,5)P₂) to phosphoinositide (3,4,5) triphosphate (PtdIns(3,4,5)P₃) in vivo, a critical second messenger involved in signal transduction. Activation of Class I PI3Ks results in the accumulation of PtdIns (3, 4, 5)P₃ on the inner leaflet of the cytoplasmic membrane. Several proteins domains, such as pleckstrin- and phox-homology domains (PH and PX, respectively) have been shown to specifically recognize PI3K-derived phosphoinositide lipid products (149, 150) and regulate the localization and activation of downstream effector molecules such as the Tec family of tyrosine kinases, PDK-1, Akt (PKB), certain isoforms of PKC, Btk, GAP/GEFs for small GTPases. In the mammalian immune system, PI3Ks are activated by a variety of receptors for antigen, cytokines, co-stimulatory
molecules, immunoglobulins, and chemo-attractants and play an important role in regulating numerous immune cell functions such as proliferation, survival, differentiation, chemotaxis, phagocytosis, degranulation, and respiratory burst (147, 148).

The Class I PI3Ks are further divided into Class IA and IB PI3Ks based on mechanism of activation and differential association with regulatory subunits. The Class IA PI3Ks exist as multiple isoforms (PI3Kα, PI3Kβ, and PI3Kδ) resulting from the association of distinct p110 catalytic subunits (p110α, p110β, and p110δ) with one of five p85/p50 regulatory subunits (p85α, p85β, p55γ, p55α, and p50α). In contrast, PI3Kγ, the only member of Class IB PI3Ks, consists of a p110γ catalytic subunit that preferentially associates a p101/p87 regulatory subunit (147, 148). Whereas Class IA PI3Ks are typically activated by receptor tyrosine kinases, activation of Class IB PI3Kγ occurs downstream of G protein coupled receptors (GPCRs).

**PI3K ACTIVATION AND REGULATION:**

The recruitment and activation of Class IA PI3Ks is mediated primarily by receptor tyrosine kinases via the association of two Src-Homology 2 (SH2) domains present within the p85 regulatory subunit to phosphorylated tyrosine resides on activated receptors or associated adaptor proteins. Tyrosine kinases phosphorylate tyrosine residues present within specific motifs within the cytoplasmic tails of activated receptors and facilitate the recruitment of Class IA PI3Ks to membrane-associated signaling complexes and their lipid substrates to promote PI3K activity. (147, 148, 151). Although Class IA PI3K activity is typically associated with the activation of receptor tyrosine kinases,
additional work has demonstrated that Class IA PI3Ks may also be activated by GPCRs, either directly by association with Gβγ proteins (p110β), or indirectly, such as through Ras (151, 152, 153). In contrast, activation of Class IB PI3Kγ is mediated primarily by G protein-coupled receptors (GPCRs) such as receptors for chemokines. PI3Kγ activity is stimulated upon ligand-receptor binding by association of the p101/p87 regulatory subunit with Gβγ subunits of trimeric G proteins.

In addition to activation by receptor associated tyrosine kinases and GPCRs, PI3K activation by small proteins of the Rho and Ras family has also been described (147). The small GTP-binding protein, Ras, has been shown to activate Class IA PI3Ks by directly binding to Class IA p110 catalytic subunits in a GTP-dependent manner (147, 154-156). Similarly, a Ras-binding domain is also present within the p110γ catalytic subunit of Class IB PI3Kγ and several studies have demonstrated Ras as an important regulator of both PI3Kγ recruitment and activation (152, 153). Ras activity is stimulated by activation of both receptor tyrosine kinases and by Gβγ subunits of activated GPCRs and therefore provides a mechanism for broad amplification of PI3K signaling downstream of a wide variety of cell receptors.

PI3K signaling is regulated primarily by the activity of the intracellular lipid phosphatases phosphatase and tensin homologue (PTEN) and Src homology 2 (SH2) domain-containing inositol phosphatase (SHIP) which function to dephosphorylate PI3K-derived PtdIns(3,4,5)P3 to generate PtdIns (4,5)P2 and PtdIns(3,4)P2, respectively (147, 157-159). PTEN acts as a direct antagonist to PI3K signaling and suppresses PI3K/Akt signaling pathways. Signaling by PTEN is known to promote cell cycle arrest and
apoptosis and to inhibit cell migration (160). Loss of PTEN function leads to the development of numerous types of cancer and autoimmunity in mice (159). Similarly, loss-of-function mutations in PTEN are associated with several human cancers including Cowden disease, Bannayan-Zonana syndrome, and Lhermitte-Duclos disease. (159). SHIP, like PTEN, typically opposes PI3K signaling to limit activation of downstream effectors, however SHIP may also provide signals to amplify PI3K signaling or may alter PI3K effector pathways by generating substrates required for the activation of different downstream effectors, such as the Tec family of kinases and activation of PLC-γ (161). Unlike PTEN, loss-of-function mutations in SHIP are rarely associated with human cancers. Rather than promoting cancer cell development, SHIP may promote cancer cell survival by generating lipid substrates that provide a docking site for the recruitment and subsequent activation of downstream effectors, including Akt (161).

**PI3K SIGNALING IN IMMUNE CELLS:**

The use of broad-spectrum PI3K inhibitors such as wortmannin and LY294002 facilitated early investigations into the biological effects of PI3K-signaling in various systems but were limited by questions regarding enzyme- and isoform- specificity. The development of p110 isoform-specific inhibitory antibodies, genetically modified mice, and more recently, the development of small molecule PI3K isoform-selective kinase inhibitors has facilitated further investigation focused on understanding the physiological role of signaling by distinct PI3K isoforms in health and disease.
CLASS IA PI3Ks: PI3Kα AND PI3Kβ:

Class IA PI3Kα and PI3Kβ are ubiquitously expressed and studies using gene-targeting of embryonic stem cells have revealed essential and non-redundant roles for p110α and p110β isoforms in embryonic growth and development, as genetic ablation of either isoform results in early embryonic lethality in mice (162, 163). Early studies into the specific role of distinct Class I PI3K isoforms involved microinjection of primary cells and numerous immortalized cell lines with PI3K isoform-specific inhibitory antibodies (164-168). Subsequent studies using gene-targeted mice and small molecule pharmacological PI3K isoform-selective inhibitors have further enhanced our current understanding of the biological effects of signaling by specific isoforms under normal conditions as well as in models of infectious and inflammatory diseases and cancer (169-173).

Class IA p110α and p110β have been shown to have both redundant and non-redundant roles in signaling depending on cellular source and context (172, 174, 175). p110α has been shown to play an important role in regulating cell growth, metabolism, and growth factor signaling in mice (171, 172, 174) and mutation of the p110α isoform is often observed in primary tumors (176).

Like p110α, p110β is involved in regulating aspects of cellular metabolism by controlling insulin sensitivity and glucose homeostasis and has also been implicated in tumorigenesis in mouse models of prostate cancer (177). Recent studies using isoform-selective inhibitors of p110α and p110β in stable cell lines expressing either PI3K isoform have identified an important and novel function for p110β in controlling DNA
replication. Signaling by PI3Kβ controls cell cycle progression into S phase, DNA elongation, and the binding of transcription factors to DNA polymerase and subsequent DNA binding and replication by regulating the activity of nuclear PKB (178).

Although Class IA PI3Ks were originally accepted to mediate signaling downstream from activated receptor tyrosine kinases, several studies have identified an additional GPCR-mediated pathway of p110β activation. In a well established cell line of embryonic fibroblasts which do not express p110γ, p110β was found to mediate Akt phosphorylation in response to stimulation with numerous GPCR-ligands including activation by stromal cell-derived factor, sphingosine-1-phosphate, and lysophosphatidic acid but not to activation with PDGF, insulin, or insulin-like growth factor 1, which are known to activate receptor tyrosine kinases to mediate Akt activity (177). Furthermore, in macrophages, p110β contributes to Akt phosphorylation induced by activation of the GPCR for C5a but does not induce phospho-Akt in response to activation by CSF-1 (179).

Although leukocytes express all Class I PI3K isoforms, Class IA PI3Kδ and Class IB PI3Kγ are predominantly expressed by cells of the immune system. Whereas genetic ablation of the p110α or p110β catalytic subunit of Class IA PI3Ks results in embryonic lethality (162, 163), p110δ and p110γ mutant mice are viable and have revealed a major role for both isoforms in mediating numerous stages and components of the host inflammatory response.
PI3Kδ AND PI3Kγ SIGNALING IN LEUKOCYTES:

The development of PI3Kδ and PI3Kγ mutant mice has clearly illustrated an important role for these PI3K isoforms in regulating numerous components of the inflammatory response including chemotaxis and cell migration, degranulation, proliferation and cytokine production by immune cells (180). The pivotal role of PI3Kδ and PI3Kγ in regulating key components of inflammatory responses has prompted a significant interest in these two isoforms as potential pharmaceutical targets for drug development against numerous inflammatory-mediated diseases including rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), allergic disorders, and respiratory diseases such as asthma and chronic obstructive pulmonary disease (COPD) (180). The following section will review the current literature regarding the activity and function of PI3Kδ and PI3Kγ in distinct immune cell populations and their role in inflammation.

NEUTROPHILS AND MACROPHAGES:

Neutrophils and macrophages play an important role in the innate immune response against microbial pathogens. Neutrophils and macrophages are rapidly recruited to the site of infection in response to microbial products and inflammatory mediators present and/or released at the site of infection. The recruitment of cells to the site of infection is dependent upon the production of inflammatory mediators, such as cytokines and chemokines that trigger chemotaxis along a chemokine gradient to the site of infection. Neutrophils and macrophages contribute to host defense against pathogens by producing reactive oxygen species and other anti-microbial products and by secreting
chemokines and cytokines to promote the recruitment of immune cells and the development of adaptive immune responses.

PI3Kδ and PI3Kγ have been shown to play an important role in mediating leukocyte migration both in vitro and in vivo. Neutrophils and macrophages obtained from PI3Kγ−/− mice are impaired in their capacity to migrate in response to stimulation with numerous chemo-attractants including fMLP, C5a, IL-8 and RANTES (180-183). Similarly, PI3Kγ-deficient animals exhibit a significant reduction in inflammatory cell infiltration in models of bacterial sepsis and treatment of wild-type mice with the PI3Kδ isoform-selective inhibitor IC87114 was found to substantially reduce cell infiltration in a murine model of pulmonary inflammation (180-184).

PI3Kδ and PI3Kγ mediate various stages of leukocyte migration, including selectin-mediated attachment, leukocyte rolling, integrin-mediated adhesion, and extravasation through the endothelial wall and into the surrounding tissue (184). Interestingly, PI3Kγ expressed by endothelial cells has also been shown to play an important role in mediating leukocyte migration by controlling selectin-mediated attachment and interaction with the inflamed vessel wall (185).

Respiratory burst, or the production of reactive oxygen species (ROS) by neutrophils is a critical component of the innate immune response and host defense against microbial pathogens. ROS mediate microbial killing both directly by the production of destructive oxygen radicals, or indirectly by activating phagosomal proteases (186, 187). Studies using human neutrophils have demonstrated a biphasic regulation of ROS production involving both PI3Kγ and PI3Kδ in the generation of
PtdIns(3,4,5)P$_3$ and subsequent ROS production in response to pro-inflammatory signals TNF-α and fMLP (186, 187). Similarly, mouse neutrophils obtained from PI3K$\gamma^{-/-}$ mice are impaired in their ability to generate ROS in response to fMLP stimulation and PI3K$\gamma$-selective inhibition by small molecule PI3K$\gamma$ inhibitors similarly prevents respiratory burst by mouse macrophages (186).

**MAST CELLS AND EOSINOPHILS:**

Mast cells are bone marrow-derived cells that reside in the tissues and play an important role in immediate hypersensitivity reactions. Mast cells express high affinity Fc$\varepsilon$ receptors (Fc$\varepsilon$Rs) and upon cross-linking of the receptor to IgE-opsonized antigen release cytoplasmic granules containing histamine and other inflammatory mediators into the tissues (169). Aberrant mast cell responses are associated with several inflammatory conditions including allergic asthma and rhinitis and anaphylaxis (169). Mast cell growth, differentiation, and activation are driven primarily by the stem cell factor (SCF) Kit-ligand and by antigen-IgE complexes binding to SCF receptors and Fc$\varepsilon$Rs, respectively, on the cell surface. Activation of both SCF and Fc$\varepsilon$ receptors is associated with the activation of tyrosine kinases and the generation of PtdIns(3,4,5)P$_3$ by Class IA PI3-kinases (169).

Early studies using broad-spectrum PI3K inhibitors wortmannin and LY294002 identified the involvement of PI3K signaling in controlling degranulation and the release of inflammatory mediators in response to Fc$\varepsilon$R activation (180, 188). Recent studies using PI3K$\delta$ mutant mice and PI3K$\delta$ isoform-selective kinase inhibitors have
demonstrated a prominent role for signaling by the Class IA p110δ isoform in controlling numerous cellular responses in mast cells including proliferation, adhesion, migration, and IgE-allergen:FceR-induced degranulation and cytokine production (169).

Bone marrow-derived precursor cells expressing a catalytically inactive form of PI3Kδ are impaired in their ability to differentiate into mast cells in response to treatment with IL-3 and SCF, growth factors known to induce mast cell differentiation in vitro. Furthermore, PI3Kδ mutant bone marrow-derived mast cells (BMMCs) as well as WT BMMCs treated with the PI3Kδ isoform-selective inhibitor IC87114 exhibit impaired degranulation downstream of FcεR activation by IgE-allergen complexes (180). In fact, BMMCs obtained from PI3Kδ mutant mice exhibit a near complete (90%) reduction in Class IA PI3K lipid kinase activity, suggesting that the p110δ isoform is almost entirely responsible for Class IA PI3K-mediated PtdIns(3,4,5)P3 production in mast cells (169). In line with these findings is the protective phenotype of PI3Kδ mutant mice observed against passive allergic anaphylaxis in mouse models of cutaneous IgE-antigen injection (169, 180, 189).

The Class IB PI3Kγ isoform has also been implicated in mediating signaling events involved in mast cell degranulation. Mast cells treated with PI3Kγ isoform selective inhibitors and mast cells derived from PI3Kγ−/− animals have demonstrated a role for signaling by the p110γ isoform in the amplification of signaling in mast cells and sustained degranulation at later stages. Unlike PI3Kδ mutant mice, PI3Kγ-deficient mast cells exhibit normal degranulation at early time points, but are reduced at later time points (169, 180). Like PI3Kδ mutant mice, PI3Kγ−/− animals are resistant against
passive systemic anaphylaxis (180, 190). Taken together, these findings confirm an important role for both PI3Kδ and PI3Kγ in regulating mast cell activity.

Eosinophils are bone marrow-derived granulocytes that serve as the major infiltrating cells in IgE-mediated allergic reactions as well important mediators of the host defense against extracellular parasites such as helminths. Furthermore eosinophils are responsible for the chronic inflammatory response and airway remodeling observed in allergic asthmatics (191). Eosinophils contribute to chronic inflammation by producing ROS, lipid inflammatory mediators, and cytokines (180). PI3K signaling is required for the release of eosinophils from the bone marrow and trafficking to the lung in allergic pulmonary inflammation, a process that is inhibited by the pan-PI3K inhibitor wortmannin (192). As with neutrophils and macrophages, eosinophil chemotaxis is severely impaired by PI3K inhibitors LY294002 and treatment with wortmannin significantly reduces eosinophilia in a mouse model of allergic asthma as well as in Guinea Pig models of allergic bronchial inflammation (193, 194).

PI3Kγ is the major Class I PI3K isoform responsible for mediating eosinophil recruitment both in vitro and in vivo. PI3Kγ−/− mice exhibit a significant reduction in airway inflammation and airway remodeling in a mouse model of eosinophil-mediated allergen-induced pulmonary inflammation (180, 191).

T CELLS AND B CELLS:

In naïve T cells, PI3-kinases promote T cell proliferation induced by CD3/TCR complex activation or stimulation with IL-2. Early studies using wortmannin and
LY294002 have demonstrated that signaling by Class I PI3-kinase is required for antigen-specific proliferation and differentiation of naïve murine CD4+ T cells as well as CD3-induced activation and IL-2 production by CD8+ T cells (180, 195, 196). The availability of genetically modified mice has greatly enhanced our understanding of the role of distinct PI3K isoforms in lymphocytes and has revealed important roles for both PI3Kδ and PI3Kγ in the development, maturation, differentiation, and activation of T cells.

A role for the Class IB PI3Kγ isoform has been identified in the development of T cells. PI3Kγ-deficiency in mice is associated with modest increases in the proportion of double-positive (CD4+CD8+) cells in the thymus and subsequent reduction in the number of peripheral T cells in PI3Kγ−/− mice (197). Activation of PI3-kinases occurs in response to activation of TCR with or without costimulation by CD28, as well as in response to treatment with IL-2, indicating a role for PI3-kinases in regulating T cell proliferation (180, 198). While PI3Kγ is involved in amplifying signaling involved in cytokine production and cell proliferation downstream of activated TCRs, PI3Kδ appears to play a predominant role in controlling T cell activation, maturation, differentiation, and effector function.

PI3Kδ is intimately involved in regulating CD4+ T cell maturation and differentiation into distinct T cell subsets. Mice expressing a loss-of-function mutation in PI3Kδ (PI3Kδ D910A/D910A; PI3Kδ mutant mice) exhibit a significant defect in differentiation and maturation of CD4+ into Th1, Th2, and regulatory T cell (Treg) cell populations (198, 199). The defect in regulatory T cell responses observed in PI3Kδ-
mutant mice is characterized by a reduction in the proportion of Tregs in the thymus and defects in Treg suppressive capacity (IL-10 production) and is associated with an increase in development of autoimmunity in mice (198, 200). In contrast to the negative effect of PI3Kδ-deficiency in autoimmune disease, the diminished Treg response observed in PI3Kδ mutant mice has been associated with conveying protection against cutaneous leishmaniasis caused by *Leishmania major* in experimental models of murine leishmaniasis (201).

Like T lymphocytes, the Class IA PI3Kδ isoform appears to be the predominant PI3K isoform responsible for regulating B cell development, survival, differentiation, and effector function (173). PI3Kδ-‘deficient’ mice exhibit a significant reduction in specific B cell compartments within the bone marrow and spleens as compared to PI3Kδ+/+ wild type mice, indicating an important role for PI3Kδ in mediating the development and maturation of B cells *in vivo*. Furthermore, B cells from PI3Kδ mutant mice exhibit impaired proliferation in response to IgM stimulation and BAFF and exhibit decreased antibody production in response to both T cell dependent- and T cell-independent antigens (173, 180). Finally, B cells obtained from PI3Kδ mutant mice are impaired in their chemotactic response to the chemokine CXCL13, suggesting a role for PI3Kδ in mediating B cell migration downstream of GPCRs (202). In contrast, genetic ablation of PI3Kγ has little effect on B cell development, survival, or effector function, suggesting that PI3Kγ is not a critical regulator of B cells *in vivo* (200, 202).
CHAPTER 2

The Role Of Phosphoinositide 3-Kinase Gamma (PI3Kγ) In The Host Immune Response To Cutaneous Leishmania mexicana Infection

2.1 EXPERIMENTAL MODEL OF CUTANEOUS LEISHMANIA MEXICANA INFECTION IN C57BL/6 MICE:

The genetic background of the host plays an important role in determining host resistance or susceptibility to Leishmania mexicana infection in mice (203). The vast majority of studies have been performed in experimental models involving subcutaneous infection with high numbers of parasites \((10^6-10^7)\) into the footpad or back rump, however numerous studies have shown that the number of parasites inoculated, as well as the route of infection, are important factors in determining disease outcome in cutaneous leishmaniasis with both Leishmania major and Leishmania mexicana (203-206). Previous studies in our lab have demonstrated that a low dose infection model involving intradermal (i.d.) inoculation of 1000 stationary phase Leishmania mexicana promastigotes into the ear dermis is sufficient to cause infection in BALB/c and C57BL/6 mice (203). We believe that a low dose ear model of cutaneous Leishmania mexicana infection more closely mimics natural infection in both the number of parasites inoculated as well as the site of infection and provides a more physiologically relevant
model for studying factors that contribute to host susceptibility or resistance to cutaneous *Leishmania mexicana* infection.

C57BL/6 mice are susceptible to low dose *Leishmania mexicana* infection and develop large lesions harboring high parasite burdens within 8-10 weeks of infection (203). In contrast to the Th1-polarized response that mediates resistance of this strain to cutaneous infection with *Leishmania major*, C57BL/6 mice exhibit a mixed Th1/Th2 phenotype characterized by the production of both Th1- and Th2-associated cytokines in response to cutaneous infection with *Leishmania mexicana* (203). Previous studies in our lab have shown that a defect in the expression of CXCR3 chemokine receptor by activated T cells contributes to susceptibility of BALB/c mice to cutaneous infection with *Leishmania major* by preventing the homing of IFN-γ-producing T cells to the site of infection and that PI3Kγ is required for efficient CXCR3 expression by activated T cells in response to *Leishmania mexicana* infection both *in vitro* and *in vivo* (207, 208) These results suggested that PI3Kγ might mediate protective immune responses against *Leishmania mexicana* by a similar mechanism.

To test the hypothesis that the Class IB PI3Kγ isoform mediated protective immune response against cutaneous *Leishmania mexicana* infection, we examined the course of a low dose *Leishmania mexicana* infection in C57BL/6 wild type (WT) and p110γ-deficient (PI3Kγ−/−) mice. C57BL/6 WT and PI3Kγ−/− mice were inoculated with 1000 stationary phase *Leishmania mexicana* promastigotes into the ear dermis and disease progression was monitored by measuring the thickness of infected ears as compared to uninfected ears at weekly intervals. As expected, C57BL/6 WT mice
developed progressive lesions over a period of 8 weeks. Interestingly, PI3Kγ−/− mice developed significantly smaller lesions as compared to their wild type (WT) counterparts (Figure 2.1 A). In addition, limiting dilution analysis of parasite burdens within the infected lesions demonstrated the presence of high parasite titers in C57BL/6 WT mice. In contrast, the ear lesions obtained from infected PI3Kγ−/− mice were found to harbor significantly fewer parasites (Figure 2.1 B) indicating an enhanced ability of PI3Kγ−/− mice to control parasite growth and reduce disease pathology associated with cutaneous *Leishmania mexicana* infection.

Previous work by Zorko *et al.* 2006 nicely characterized the immuno-phenotype of *Leishmania mexicana*-infected C57BL/6 WT and PI3Kγ−/− mice with regards to Th1/Th2 T cell responses in an initial attempt to uncover the mechanisms involved in mediating the increased resistance observed in PI3Kγ−/− mice. Interestingly, PI3Kγ−/− mice were not found to exhibit a distinct Th1/Th2 phenotype by *Leishmania mexicana* antigen (LmAg)-stimulated draining lymph node cells *ex vivo* and like their PI3Kγ+/+ WT counterparts, produced both Th1-associated cytokines IL-12 and IFN-γ as well as Th2-associated cytokines IL-4 and IL-10. Analysis of IgG1 and IgG2a titers from sera of infected C57BL/6 WT mice revealed high antibody titers of IgG isoforms belonging to each class, reflecting the lack of Th1/Th2 bias observed in draining lymph node cells. PI3Kγ−/− animals were significantly impaired in their antibody response and exhibited low levels of both IgG1 and IgG2a as compared to controls. As antibodies are typically associated with disease exacerbation in cutaneous leishmaniasis, the diminished antibody
response observed in PI3Kγ−/− animals may contribute to the increased resistance observed in these animals.

These initial studies provided impetus to investigate the mechanisms by which PI3Kγ mediates susceptibility to *Leishmania mexicana* infection in C57BL/6 mice. Using a low dose model of cutaneous *Leishmania mexicana* infection in a mouse strain that is genetically susceptible to infection and exhibits a mixed Th1/Th2 phenotype similar to that observed in humans should provide a better model for understanding the complexities of immune regulation in human cutaneous disease.

### 2.2 PI3Kγ SIGNALING IN MACROPHAGES AND NEUTROPHILS:

PI3Kγ is a Class I PI3K preferentially expressed by cells of the immune system and consists of a p110γ catalytic subunit that associates with a p101/p87 regulatory subunit. PI3Kγ functions to phosphorylate the 3′-hydroxy position of inositol-containing phospholipids in the membrane to generate PtdIns(3,4,5)P3, an important second messenger in intracellular signal transduction, on the inner leaflet of the cytoplasmic membrane. PI3Kγ activation occurs downstream of G-protein coupled receptors (GPCRs) and has been well characterized as an important mediator of leukocyte chemotaxis (147). Activation of chemokine receptors leads to the dissociation of Gβγ subunits from heterotrimeric G protein complexes and the subsequent activation of numerous effectors involved in cell migration including PI3Ks, the Rho GTPases Rac and Cdc42, p-38 mitogen activated protein (MAP) kinase, protein kinase Cζ (PKCζ), and cytosolic phospholipase A2 (cPLA2) (181, 182, 197, 209).
The process of chemotaxis requires that cells reorganize their cytoskeleton to allow for physical changes necessary for cell migration in response to chemokine-receptor interaction. Early studies using the pan-PI3K inhibitor wortmannin clearly demonstrated a role for PI3K signaling in mediating leukocyte chemotaxis however the specific PI3K isoform(s) responsible remained unclear. Recent studies however, have identified the Class IB PI3Kγ as an important mediator of leukocyte chemotaxis both in vitro and in vivo (181, 182, 197).

Macrophages and neutrophils obtained from p110γ−/− mice show reduced migration in response to stimulation with numerous chemo-attractants including fMLP, C5a, RANTES, and IL-8 (181, 182, 197). Signaling by PI3Kγ is responsible for generating PtdIns(3,4,5)P3 downstream of GPCRs and is required for neutrophil chemotaxis and super oxide production in response to signaling by chemokines (182, 197). PI3K signals with Rac and Cdc42 to induce F-actin polymerization and cytoskeletal reorganization in response to GPCR activation (210-214). Studies in mice carrying a knock-in allele of a membrane-bound mutant PI3Kγ demonstrate that PI3Kγ signaling along with Rac is responsible for driving cell polarization by controlling the local accumulation of PtdIns(3,4,5)P3 at the leading edge (210). Neutrophils and macrophages from PI3Kγ mutant mice display uncoupled PtdIns(3,4,5)P3 production and a loss of directionality during chemotaxis in vivo (210).

Early studies using pan-PI3K inhibitors wortmannin and LY294002 demonstrated that signaling by Class I PI3Ks was similarly involved in the process of macropinocytosis and phagocytosis by macrophages, processes which are also dependent upon localized F-
actin polymerization and reorganization of the cytoskeleton (215-217). In these studies, PI3K activity was not found to be required for actin polymerization and extension of the plasma membrane and the formation of phagocytic cups, but instead was required for closure of these cups into intracellular vesicles and completion of phagocytosis by macrophages (215-217).

Studies using wortmannin and LY294002 have illustrated a role for host PI3Ks in mediating the entry of *Trypanosoma cruzi*, an obligate intracellular protozoan parasite and causative agent of Chagas’ disease in humans, into host cells *in vitro* (218-220). Wortmannin was found to significantly reduce parasite entry into macrophages and inhibit the production of PI3K-derived phospholipids induced by *Trypanosoma cruzi* infection (218). Similarly, wortmannin and LY294002 were found to inhibit *Trypanosoma cruzi* entry into both phagocytic and non-phagocytic cell lines in a dose-dependent manner (219).

A study by Gagnon *et al.* 2002 demonstrated that the endoplasmic reticulum provides a source of membrane during phagocytosis by macrophages and that signaling by PI3K is required for ER-mediated phagocytosis of inert particles as well as the intracellular parasite *Leishmania donovani* (221). Treatment of macrophages with pan-PI3K inhibitors 3-methyladenine and wortmannin were found to inhibit ER-mediated phagocytosis of latex beads and reduce uptake of *Leishmania* parasites into macrophages *in vitro*, suggesting an important role for host PI3Ks in facilitating parasite entry into host cells to promote the establishment of successful infection. These findings led us to hypothesize that by initiating actin polymerization and cytoskeletal re-arrangement
PI3Kγ may mediate susceptibility to *Leishmania mexicana* infection by facilitating phagocytic uptake of *Leishmania* parasites into macrophages and/or neutrophils.

Activation of the Class IB PI3Kγ occurs downstream from GPCRs and generates PtdIns(3,4,5)P₃ and the activation of Akt/PKB (147). PI3Kγ, along with PI3Kδ (a Class IA PI3K isoform also preferentially expressed by immune cells), signal small G proteins Rac and Cdc42 to induce F-actin polymerization and accumulation at the leading edge to drive cell migration in response to chemotactic stimuli (147, 181, 182). Because actin polymerization and cytoskeletal reorganization are also required for the process of phagocytosis, we hypothesized that PI3Kγ may mediate phagocytosis and parasite entry into host cells to promote establishment of successful infection.

To examine the specific role of PI3Kγ from that of the other Class I PI3K isoforms in controlling phagocytosis, we used the small molecule, isoform-selective inhibitor AS-605240 (5-quinoxalin-6-ylmethylene-thiazolidine-2,4-dione) to selectively inhibit PI3Kγ activity both in vitro and in vivo (222). AS-605240 successfully outcompetes ATP for its binding pocket on the enzyme, rendering the kinase inactive (222). Crystallographic structural analysis revealed that the thiazolidinedione nitrogen and the quinoxaline ring of AS-605240 interact with specific lysine and valine residues, respectively on PI3Kγ to prevent ATP binding and subsequent activation (222). Biochemical analysis revealed no effect of AS-605240 on unrelated receptors, enzymes, or ion channels when used to inhibit PI3Kγ activity at concentrations up to 10 µM (222).

In order to determine whether the Class IB PI3Kγ isoform was involved in mediating parasite entry into host cells, we examined the effect of PI3Kγ blockade on
parasite uptake into phagocytes in vitro. C57BL/6 bone marrow-derived macrophages (BMDMs), polymorphonuclear cells (PMNs/neutrophils), and human monocyte-derived macrophages (HMDMs) were treated with 1.25 µM AS-605240 or DMSO vehicle and infected with CFSE-labeled *Leishmania mexicana* promastigotes or axenic amastigotes at 33°C with 5% CO₂ for various time points. Following infection, cells were washed, and extracellular parasites were labeled using an anti-*Leishmania* spp. lipophosphoglycan (LPG) specific antibody followed by an Alexa Fluor® 594- conjugated secondary antibody for detection. Host cells were stained using DAPI. The cover slips were mounted on glass slides and analyzed by fluorescence microscopy (Figure 2.2 A-D) to quantify intracellular parasites in each group.

BMDMs, PMNs, and HMDMs treated with AS-605240 showed a significant reduction in the number of intracellular parasites as compared to mock-treated controls. (Figure 2.3 A-C). AS-605240 was also found to inhibit the uptake of *Leishmania mexicana* axenic amastigotes into C57BL/6 BMDMs in vitro (Figure 2.3 D). The inhibitory effect of AS-605240 on parasite entry into C57BL/6 BMDMs and PMNs in vitro was further confirmed using flow cytometry (Figure 2.4 A-C).

Similar results were observed using cell lines derived from bone marrow-derived macrophages isolated from neonatal C57BL/6 WT and P3Kγ⁻/⁻ mice. Macrophages derived from PI3Kγ⁻/⁻ mice and infected with *Leishmania mexicana* promastigotes in vitro contained significantly fewer parasitized macrophages as compared to PI3Kγ⁺/⁺ WT-derived macrophages (Figure 2.5). Furthermore, AS-605240 was not found to have any effect on the expression of CR3/1 or Fcγ receptors, phagocytic receptors responsible
for mediating *Leishmania* spp. entry into macrophages (Figure 2.6 A-C). Taken together, these studies suggest that signaling by the Class IB PI3Kγ isoform is important for mediating *Leishmania mexicana* entry into macrophages and neutrophils *in vitro*.

To investigate whether PI3Kγ was responsible for *Leishmania mexicana* uptake into phagocytes specifically, we examined the effect of PI3Kγ inhibition on phagocytosis of collagen-coated latex fluorobeads. As seen with *Leishmania* parasites, C57BL/6 BMDMs treated with AS-605240 to inhibit PI3Kγ activity showed greater than 50% reduction in the percentage of cells harboring intracellular beads as compared to vehicle treated controls (Figure 2.7).

The effect of AS-605240 on parasite uptake into phagocytes *in vivo* was examined using an air pouch model of cellular trafficking (223, 224). Dorsal subcutaneous pouches were created on the shaven rumps of C57BL/6 mice by injection with 2 mL of sterile air. The pouches were injected with either LPS for 3 hours or thioglycollate for 96 hours to induce infiltration of neutrophils or macrophages, respectively. These pouches were subsequently injected with CFSE-labeled *Leishmania mexicana* promastigotes along with AS-605240 (15 mg/kg) or vehicle for 1-3 hours. The cells were isolated from the pouches by lavage and stained for macrophage or neutrophil surface markers CD68 and GR1 for quantification of parasitized macrophages (CD68+/CFSE+) or neutrophils (GR1+/CFSE+) by flow cytometry. C57BL/6 mice treated with AS-605240 to inhibit PI3Kγ activity contained fewer parasitized macrophages and neutrophils in their dorsal pouches as compared to untreated (vehicle) controls (Figure 2.8 A, B). Similar results were observed for macrophages treated with AS-605240 and infected with *Leishmania*.
mexicana axenic amastigotes (Figure 2.8 C), as well as IgG-opsonized Leishmania mexicana amastigotes (Figure 2.8 D), the physiologically relevant form of the parasite found within the mammalian host. The effect of AS-605240 on amastigotes uptake by neutrophils was not investigated as neutrophils are known to interact with Leishmania parasites early in the acute phase of infection in vivo (77).

In order to confirm that the Class IB PI3Kγ isoform was in fact responsible for mediating parasite uptake specifically, we evaluated the effect of small molecule kinase inhibitors specific for individual Class I PI3K isoforms (PI3Kα, PI3Kβ, PI3Kδ, and PI3Kγ) on the uptake of Leishmania mexicana promastigotes into macrophages in vitro. PI3Kα, PI3Kβ, PI3Kδ, and PI3Kγ activity was inhibited in ANA-1 macrophages in vitro using the isoform-selective inhibitors PI3-kinase alpha Inhibitor 2, TGX-221, IC-87114, and AS-605240, respectively. ANA-1 macrophages were treated with either a Class I PI3K isoform-selective inhibitor or vehicle and infected with CFSE-Leishmania mexicana promastigotes in vitro at 33°C/5% CO₂ and analyzed after 1-3 hours for quantification of parasitized macrophages by flow cytometry (Figure 2.9). Only macrophages treated with AS-605240 (Figure 2.9 B) exhibited a substantial reduction in the percentage of parasitized cells as compared to vehicle-treated controls (Figure 2.9 A). Inhibition of Class IA PI3Kα, PI3Kβ, or PI3Kδ had little effect on parasite uptake into ANA-1 macrophages in vitro (Figure 2.9 C-E). These findings suggest that parasite entry into macrophages and neutrophils is mediated primarily by the Class IB PI3Kγ isoform and occurs largely independent of signaling by Class IA PI3K isoforms PI3Kα, PI3Kβ, and PI3Kδ.
2.3 DISCUSSION:

Phagocytosis and macropinocytosis are processes by which large particles or fluids, respectively, are internalized into cells (225). Phagocytosis is a receptor-mediated process involving specific cell surface receptors such as TLRs, receptors for complement, and Fc receptors expressed on the surface of macrophages, neutrophils, and dendritic cells. The binding and activation of phagocytic receptors induces the clustering of cell surface receptors and downstream signaling complexes into cholesterol-rich microdomains in the plasma membrane. Both phagocytosis and micropinocytosis are processes that generate intracellular vesicles resulting from invaginations of the plasma membrane and the subsequent closure of phagocytic ‘cups’ induced upon receptor-ligand interaction and/or activation (226). Macrophages and neutrophils are remarkable in their ability to phagocytose extremely large particles, often much larger in diameter than themselves. Phagocytosis of such large particles requires additional sources of plasma membrane, which may be acquired from recycling of (late) endosomes, lysosomes, specific granules, and/or the endoplasmic reticulum (ER) (221, 226, 227).

Early studies using pan-PI3K inhibitors such as wortmannin and LY294002 demonstrated a role for signaling by Class I PI3Ks and the production of PtdIns(3,4,5)P₃ in phagocytosis and macropinocytosis by macrophages and neutrophils (215-217, 226, 228). Treatment of macrophages and neutrophils with PI3K inhibitors abolishes PtdIns(3,4,5)P₃ accumulation and prevents phagocytosis. Similarly, macrophages genetically deficient or expressing a catalytically inactive allele of SHIP, a phosphatase that localizes to forming phagosomes and limits PtdIns(3,4,5)P₃ accumulation by
dephosphorylating \( \text{PtdIns}(3,4,5)P_3 \) to \( \text{PtdIns}(3,4)P_2 \) \textit{in vivo}, exhibit enhanced phagocytosis (229).

Whereas 3’ phosphoinositides are essentially undetectable in resting cells, phagocytosis is associated with the rapid accumulation of \( \text{PtdIns}(3,4,5)P_3 \) at the site of receptor-ligand interaction. \textit{In vivo} live cell imaging using GFP-fusions to the PH-domain of Akt, Gab1, and Btk (substrates that bind \( \text{PtdIns}(3,4,5)P_3 \) \textit{in vivo}) expressed in murine macrophages has revealed that \( \text{PtdIns}(3,4,5)P_3 \) rapidly accumulates in response to presentation with IgG-coated sheep red blood cells and that the production of \( \text{PtdIns}(3,4,5)P_3 \) is exquisitely localized to the region of the ‘phagosomal cup’, with little-to-no diffusion throughout the plasma membrane (230). PI3K and the GTPases Cdc42, Rac1, and Arf6 are active within the leading edge of forming ‘cups’ and are believed to mediate signaling to induce local actin polymerization surrounding the ingested particle.

Phagocytosis involves the local accumulation of polymerized actin filaments that drive pseudopod formation and extension of the plasma membrane as well as the contraction and closure of the distal portion of the plasma membrane to form complete, membrane-bound phagocytic vesicles containing the ingested particle (226-228). The production of \( \text{PtdIns}(3,4,5)P_3 \) is rapid albeit transient, and is present during early stages of pseudopod extension but disappears within 2-3 minutes of phagosome closure (230). The loss of \( \text{PtdIns}(3,4,5)P_3 \) coincides with the accumulation of SHIP on the phagosomal membrane and the disassembly of phagosomal F-actin during later stages of phagosome maturation.
The role of PI3Ks in actin polymerization during phagocytosis is somewhat controversial. In several studies, PI3K signaling was not found to be required for early receptor/ligand-induced actin polymerization, but was instead required for contraction of the plasma membrane and closure of phagocytic ‘cups’ into membrane-bound vesicles (215, 230, 231). Video microscopy analysis revealed that inhibition of PI3K activity by wortmannin or LY294002 resulted in the formation of incomplete, ‘open’ phagosomes, in which the plasma membrane did not seal to form intact membrane-bound vesicles, suggesting that PI3K activity was involved in the later stages of phagocytosis by facilitating phagosome closure (215, 231). Interestingly, work by Cox et al. 1999 suggested that PI3K activity is also involved in the early stages of phagocytosis of large particles and is required for facilitating the acquisition of additional sources of plasma membrane from endocytic vesicles for formation of phagocytic vacuoles by macrophages (229). Although the precise role of PI3Ks in inducing actin polymerization remain unclear, it is clear that PI3Ks are important for regulating signaling to the actin cytoskeleton during phagocytosis.

PI3Kγ is preferentially expressed by immune cells including macrophages and neutrophils and is an important mediator of leukocyte chemotaxis. Neutrophils and macrophages from PI3Kγ−/− mice fail to produce PtdIns(3,4,5)P₃ and are severely impaired in their ability to migrate in response to chemotactic stimuli in vitro (197). Similarly, neutrophils and macrophages from PI3Kγ-mutant mice display uncoupled PtdIns(3,4,5)P₃ production and a loss of directionality during chemotaxis in vivo (210). The production of PtdIns(3,4,5)P₃ in response to GPCR ligands such as fMLP and C5 is
dependent upon PI3Kγ, and PI3Kγ-mediated PtdIns(3,4,5)P3 production at the leading edge has been shown to mediate neutrophil migration by regulating process such as cell adhesion and the local accumulation of polymerized actin at the leading edge (232).

We found that in addition to mediating leukocyte chemotaxis, PI3Kγ is involved in phagocytosis and facilitating the entry of *Leishmania mexicana* into macrophages and neutrophils. Pharmacological inhibition of PI3Kγ by AS-605240 was found to significantly reduce the uptake of *Leishmania mexicana* parasites into macrophages and neutrophils both *in vitro* and *in vivo*. The use of a small molecule inhibitor however, did not exclude the possibility that the defect in phagocytosis observed in AS-605240-treated cells was the result of non-specific inhibition of other PI3K isoforms or unrelated enzymes and not of specific inhibition of PI3Kγ. Using macrophages derived from neonatal C57BL/6 WT and PI3Kγ−/− mice, we confirmed that parasite entry into macrophages was in fact mediated by PI3Kγ. Like AS-605240-treated macrophages, macrophages derived from PI3Kγ−/− mice and infected with *Leishmania mexicana* promastigotes were impaired in their ability to internalize parasites and harbored fewer intracellular parasites as compared to PI3Kγ+/+ WT macrophages.

Signaling by PI3Kγ does not appear to be specific for the uptake of *Leishmania* parasites, as treatment of macrophages and neutrophils with AS-605240 to inhibit PI3Kγ activity were similarly impaired in their ability to phagocytose collagen-coated latex beads. AS-605240 was not found to have any effect on the expression of *Leishmania* spp. phagocytic receptors CR3/CR1 or FcγRs on the surface of macrophages, further supporting that the reduced parasite uptake observed in the absence of PI3Kγ signaling
was not due to altered expression of receptors involved in parasite binding, but rather a direct effect of PI3Kγ signaling during phagocytosis and in facilitating the uptake of *Leishmania* parasites into macrophages and neutrophils.

PI3Kγ appears to be the predominant PI3K isoform responsible for mediating the entry of *Leishmania* parasites into macrophages. Small molecule inhibitors of PI3Kα, PI3Kβ, and PI3Kδ had no effect on uptake of *Leishmania mexicana* promastigotes into macrophages *in vitro*. Only specific inhibition, or genetic-deficiency of PI3Kγ resulted in parasite uptake by macrophages and neutrophils, suggesting that the PI3Kγ is a major regulator of phagocytosis and parasite uptake into host phagocytes. These findings suggest that targeting PI3Kγ using isoform-selective PI3Kγ inhibitors may be a therapeutically viable strategy for treating *Leishmania mexicana* infection and possibly infections with other intracellular pathogens of that target host phagocytes.

2.4 MATERIALS AND METHODS:

ANIMALS:

Eight-10–week old C57BL/6 mice (female) were purchased from Harlan® (Harlan®, Indianapolis, Indiana, USA). PI3Kγ-deficient C57BL/6 mice (back crossed 10 times) were originally a generous gift of Dr. Bao Lu. C57BL/6 wild type (WT) and C57BL/6 PI3Kγ−/− mice were maintained in a pathogen free animal facility at The Ohio State University in accordance with National and Institutional guidelines.
PARASITES:

*Leishmania mexicana* (MNYC/BZ/62/M379) was maintained by serial passage of amastigotes inoculated subcutaneously (s.c.) into the shaven rumps of C57BL/6 mice. Amastigotes were obtained from the infected lesions and grown *in vitro* in M199 media (GIBCO® Invitrogen™ Carlsbad, California, USA) supplemented with 10% FBS and 1% Penicillin/Streptomycin (GIBCO® Invitrogen™ Carlsbad, California, USA) at 26°C without CO₂ to generate *Leishmania mexicana* promastigotes for both *in vitro* and *in vivo* studies. *Leishmania mexicana* axenic amastigotes were grown *in vitro* from promastigotes cultured in Schneider’s *Drosophila* media (GIBCO® Invitrogen™ Carlsbad, California, USA) pH=5.2 supplemented with 10% FBS, 1% Penicillin/Streptomycin at 33°C/5%CO₂ for 10-14 days.

INFECTION PROTOCOL:

Metacyclic promastigotes for infections were obtained from stationary phase promastigotes by negative selection by agglutination with peanut lectin. Early passage *Leishmania mexicana* promastigotes were washed in Hanks Balanced Salt Solution (HBSS, 1X) (GIBCO® Invitrogen™ Carlsbad, California, USA) 3X by centrifugation at 3000 RPM for 5 minutes at 4°C and suspended at a concentration of 1.5 x 10^8 parasites/mL. Lectin from *Arachis Hypogaea* (peanut) (Sigma® Aldrich, St. Louis, Missouri, USA) was added to the parasite suspension (75 µg/mL; final concentration) and incubated at room temperature for 30 minutes. Peanut lectin-agglutinated parasites were centrifuged at 200 RCF for 5 minutes at 4°C and the metacyclic (non-agglutinated)
promastigotes were collected from the supernatant and re-suspended in RPMI cell culture media (GIBCO® Invitrogen™ Carlsbad, California, USA) supplemented with 10% FBS and 1% Penicillin/Streptomycin (GIBCO® Invitrogen™ Carlsbad, California, USA). Experimental animals were infected intradermal (i.d.) into the left ear dermis with $1 \times 10^3$ PNA-selected *Leishmania mexicana* promastigotes.

**LESION DEVELOPMENT:**

Disease progression was monitored at weekly intervals by measuring the diameter of the infected ear minus the diameter of the uninfected ear. The ears were measured using a Mitutoyo Pocket Thickness Gage (Mitutoyo American Corporation, Aurora, Illinois, USA). Data is presented as mean lesion size in millimeters +/- standard error from 3 independent experiments (n= 15 mice per group).

**PARASITE BURDENS:**

Parasite burdens within the infected ears were determined by limiting dilution analysis. The infected ears were removed, separated, and passed through a 70 µm nylon cell strainer (BD Falcon™, Bedford, Massachusetts, USA). Cells were washed in PBS and serially diluted (1/10 dilutions) across a Costar® 96-well flat bottom tissue culture plate (Corning Incorporated, Corning, New York, USA) in M199 cell culture media in duplicate and incubated at 26°C without CO$_2$ for 5-10 days. At this time the greatest dilution yielding viable parasites was recorded for each ear. Data presented as mean
parasite dilution +/- standard error from 2-3 independent experiments (n = 10-15 mice per group).

**CELL CULTURE AND ISOLATION:**

Bone marrow-derived macrophages (BMDMs) were obtained from bone marrow-derived (BMD) cells isolated from the femurs and tibias of C57BL/6 wild type mice. BMD cells were cultured in RPMI 1649 cell culture media (GIBCO® Invitrogen™, Carlsbad, California, USA) supplemented with 10% FBS and 1% Penicillin/Streptomycin, and 20% supernatants from *in vitro*-cultured L929 cells at 37°C and 5% CO\(_2\) for 7-10 days in vented 75 cm\(^2\) CELLSTAR® cell culture flasks (Greiner Bio-One, Frickenhausen, Germany).

Polymorphonuclear cells (PMNs) were obtained from the peritoneal exudates of C57BL/6 mice. C57BL/6 mice were injected i.p. with LPS (2 µg/mL) to induce neutrophil recruitment. Three hours later, the mice were sacrificed and cells were isolated from the peritoneum by lavage. PMNs were cultured in RPMI 1640 cell culture media supplemented with 10% FBS and 1% Penicillin/Streptomycin on collagen-coated cover slips or tissue culture plates at 37°C and 5% CO\(_2\).

Peripheral blood mononuclear cells (PBMCs) were obtained from fresh source leukocytes using the ACCUSPIN™ System-Histopaque®-1077 density gradient (Sigma-Aldrich®, Saint Louis, Missouri, USA). Monocyte-derived macrophages were generated by plating
2 x 10^7 cells in RPMI 1690 cell culture media without FBS. Cells were allowed to adhere for 2 hours at which point non-adherent cells were washed with PBS and removed by aspiration. The remain cells were cultured in RPMI 1640 cell culture media supplemented with 10% FBS and 1% Penicillin/Streptomycin at 37°C/5% CO_2 for 5 days to obtain human monocyte-derived macrophages (HMDMs).

ANA-1 and PI3Kγ−/− macrophage cells lines were a generous gift from Dr. Danuta Radzioch from McGill University, Montreal, Quebec. ANA-1 and PI3Kγ−/− macrophage cells lines were grown in 75 cm^2 tissue culture plates in High Glucose Dulbecco’s Modified Eagle Media (DMEM) (GIBCO® Invitrogen™, Carlsbad, California, USA) supplemented with 20% FBS and 1% Penicillin/Streptomycin at 37°C/5% CO_2.

**ANTIBODIES AND REAGENTS:**

*Leishmania mexicana* parasites were fluorescently labeled with 5(6)-carboxyfluorescin diacetate N-succinimimidyl ester (CFSE) (Fluka BioChemika, Buchs, Switzerland) for all uptake studies. Parasites were washed in 1X PBS (GIBCO® Invitrogen™, Carlsbad, California, USA), re-suspended at concentration = 1-10 x 10^6/mL, and labeled with CFSE (1:1000) at 37°C for 20 minutes. Extracellular parasites were labeled using an anti-*Leishmania* lipophosphoglycan (LPG) antibody (Cederlane® Manufactured Immunology Reagents, Ontario, Canada) followed by an Alexa Fluor® 594-conjugated secondary antibody (Molecular Probes® Invitrogen™, Carlsbad, California, USA) for detection. Host cells were labeled with 4,6-diamidino-2-phenylindole (DAPI) using ProLong®
Gold antifade reagent with DAPI (Molecular Probes® Invitrogen™, Carlsbad, California, USA). PE-conjugated anti-CD68 and PE-conjugated anti-GR1 (Biolegend®, San Diego, California, USA) was used to quantify infected macrophages and neutrophils by flow cytometry. Flow cytometric analysis of expression of macrophage phagocytic receptors CD11b, CD18, and CD16/32 was performed using PE-conjugated anti-CD11b, FITC-conjugated anti-CD18, and APC-conjugated anti-CD16/32 antibodies (Biolegend®, San Diego, California, USA). Collagen-coated FluoroSpheres® (Invitrogen™, Carlsbad, California, USA; 2 µM) were used for analysis of non-specific phagocytosis by macrophages.

**MICROSCOPY EQUIPMENT:**

Fluorescence microscopy images were acquired using a Carl Zeiss Axio Observer D1® inverted epi-fluorescence microscope (Carl Zeiss MicroImaging, LLC, Thornwood, New York, USA) equipped with a 20X Zeiss Plan Neofluar® objective (numerical aperture 0.5). Illumination was provided by a xenon arc lamp (Sutter Instrument, Novato, California, USA) using filter sets from Chroma Technology Corporation, Bellow Falls, Vermont, USA: DAPI (49000), GFP (49002), and DSRED (49005). Images were acquired using a Cascade® II: 512 camera (Photometrics™, Tucson, Arizona, USA). Image acquisition and analysis carried out by Metamorph® Imaging and Analysis System software (Universal Imaging, Molecular Devices®, Sunnyvale, California, USA).
**IN VITRO PARASITE UPTAKE ASSAY:**

For fluorescence microscopy studies, 3.5-5 x 10^5 BMDMs, PMNs, or HMDMs were cultured on 1.5 mm glass cover slips at 37°C/5% CO\(_2\) and allowed to adhere overnight. Adherent cells were treated with 1.25 µM AS-605240 or sterile DMSO vehicle 30 minutes prior to infection. Cells were infected with CFSE-labeled parasites at a ratio of 7:1 parasites: cells for 0-3 hours at 33°C/5% CO\(_2\). Following infection, cells were washed to remove media, excess drug, and free parasites, fixed in 2% PFA, stained, and mounted on glass slides for imaging. A minimum of 20 images (each containing ~50-300 cells) was obtained for each group. For flow cytometric quantification of parasite uptake, cells were plated in 12 or 24 well tissue culture plates and infected with CFSE-labeled parasites or FITC-labeled latex beads at a ratio of 7:1 parasites/beads: cells in the presence or absence of 1.25 µM AS-605240 for 1-3 hours at 33°C/5% CO\(_2\). Cells were washed and stained with PE-conjugated anti-CD68 or PE-conjugated GR1 for quantification of parasitized macrophages or neutrophils, respectively.

**COMPARATIVE PI3K STUDIES:**

An immortalized macrophage cell line derived from C57BL/6 mice (ANA-1) was used for comparative studies of different Class I PI3K isoforms in parasite uptake. ANA-1 macrophages were grown in 12-well tissue culture plates at 37°C/5% CO\(_2\) and allowed to adhere overnight. Adherent cells were treated with either PI3-Kinase α Inhibitor 2 (10 nM; Cayman Chemical, Ann Arbor, Michigan, USA), TGX-221 (10 nM; Cayman
Chemical, Ann Arbor, Michigan, USA), IC87114 (1 µM; Selleck Chemicals, LLC, Houston, Texas, USA), AS-605240 (1.25 µM) or DMSO vehicle 30 minutes prior to infection with CFSE-labeled *Leishmania mexicana* promastigotes. Infected cells were incubated at 33°C/5% CO₂ for 1 hour. Following infection, cells were washed to remove excess media, drug, and unbound parasites and analyzed by flow cytometry for quantification of parasitized (CFSE+) cells. All assays were performed at a ratio of 7:1 parasites: cells.

**IN VIVO PARASITE UPTAKE ASSAY:**

Dorsal subcutaneous pouches were created on the shaven backs of C57BL/6 wild type mice by injection with 3 mL of sterile air. These pouches were injected with either thioglycollate (2 mL) or LPS (20 µg/mL) to recruit macrophages or neutrophils into the pouches, respectively. Ninety-six hours (for macrophage studies) or 3 hours (for neutrophil studies) later, these pouches were infected with 4 x 10⁶ CFSE-labeled *Leishmania mexicana* parasites in the presence of either AS-605240 (15 mg/kg) or vehicle for 1-3 hours. Following infection, mice were sacrificed and cells isolated from the pouches by lavage. Cells were washed and stained for flow cytometric analysis of infected macrophage (CD68+/CFSE+) or neutrophil (GR1+/CFSE+) cell populations.
STATISTICAL ANALYSIS:

Statistical significance was determined using an unpaired Student’s $t$ test. For limiting dilution analysis of parasite burdens, statistical significance was determined by a Mann Whitney $U$ prime test.
Figure 2.1. Effect of PI3Kγ-deficiency on disease progression and pathogenesis of cutaneous *Leishmania mexicana* infection. C57BL/6 mice genetically deficient of the p110γ subunit of PI3Kγ (PI3Kγ−/−) were infected by inoculating 1x 10⁴ stationary phase *Leishmania mexicana* promastigotes into the left ear dermis. (A) Disease progression was monitored by measuring the thickness of infected ears using a dial-gauge micrometer at weekly intervals. Data are expressed as increase in thickness of infected ears as compared to uninfected ears. Data shown are from 3 independent experiments (n= 15 mice per group) and are presented as mean lesion size (mm) +/- standard error. For lesion sizes, * indicates p< 0.05 as determined by an unpaired Students’ t-test. (B) Parasite burdens at weeks 3, 6, and 9 post-infection as determined by limiting dilution analysis. Data are expressed as mean log parasite dilution +/- standard error and are representative from 3 independent experiments with similar results. For parasite burdens, * indicates p< 0.05 as determined by the Mann-Whitney *U* prime test.
Figure 2.1

A. Lesion Diameter

B. Parasite Burdens

* p < 0.05
Figure 2.2. Fluorescence microscopy analysis effect of AS-605240 on *Leishmania mexicana* uptake into phagocytes *in vitro*. C57BL/6 wild type BMDMs (A), PMNs (B), and human monocyte-derived macrophages (HMDMs) (C) were plated on glass cover slips and infected with CFSE-labeled *Leishmania mexicana* promastigotes in the presence of 1.25 µM AS-605240 or DMSO vehicle at 33°C/5% CO₂ for 1-3 hours. (D) C57BL/6 BMDMs were plated on glass coverslips and infected with CFSE-labeled *Leishmania mexicana* axenic amastigotes in the presence of AS-605240 or vehicle. All experiments were performed at a ratio of 7:1 parasites: cells. Following infection, cells were washed with PBS to remove excess drug, fixed in 3% paraformaldehyde (PFA), and extracellular parasites were labeled using an anti-*Leishmania* LPG specific primary antibody followed by an Alexa Fluor® 594 conjugated secondary antibody for detection. Images were acquired using a Carl Zeiss Axio Observer D1® inverted epi-fluorescence microscope (20X).
Figure 2. Continued

A. BMDMs

Vehicle

AS-605240

B. PMNs

Vehicle

AS-605240
Figure 2.2 Continued
Figure 2.3. AS-605240 reduces phagocytosis of *Leishmania mexicana* by macrophages and neutrophils *in vitro*. Quantification of intracellular promastigotes in C57BL/6 BMDMs (A), PMNs (B), and HMDMs (C) by fluorescence microscopy. Quantification of intracellular axenic amastigotes in BMDMs (D) by fluorescence microscopy. Cells were plated on glass cover slips and treated with 1.25 µM AS-605240 or DMSO prior to infection with CFSE-labeled *Leishmania mexicana* parasites. A minimum of 1000 cells were enumerated for each time point. Data are expressed as mean number of intracellular parasites per 100 cells +/- standard error from 2 independent experiments. * indicates p< 0.05 as determined by an unpaired Students’ *t* test.
Figure 2.

A. BMDM/promastigotes

B. PMN/promastigotes

C. HMDM/promastigotes

D. BMDM/amastigotes

* p<0.05
Figure 2.4. Effect of AS-605240 on phagocytosis of *Leishmania mexicana* by macrophages and neutrophils *in vitro*. Quantification of parasitized macrophages and neutrophils by flow cytometry. C57BL/6 BMDMs (A) and PMNs (B) were treated with 1.25 µM AS-605240 or DMSO vehicle 30 minutes prior to infection with CFSE-labeled *Leishmania mexicana* promastigotes at 33°C/5% CO₂ for 1 hour. Following infection, cells were washed with PBS to remove excess drug and extracellular parasites and stained for flow cytometric analysis of parasitized (A) macrophages (CD68+/CFSE+) and (B) neutrophils (GR1+/CFSE+) cell populations. C57BL/6 BMDMs were treated with AS-605240 or DMSO and infected with CFSE-labeled *Leishmania mexicana* axenic amastigotes (C) at 33°C/5% CO₂ for 1 hour. Cells were washed and stained for flow cytometric analysis of parasitized macrophage (CD68+/CFSE+) cell populations. Data presented are representative of 1 of 3 independent experiments with similar results.
Figure 2.4

A. Vehicle  

CFSE- *L. mexicana* promastigotes

AS-605240

B. Vehicle  

CFSE- *L. mexicana* promastigotes

AS-605240

C. Vehicle  

CFSE- *L. mexicana* amastigotes

AS-605240
Figure 2.5. PI3Kγ mediates phagocytosis by macrophages in vitro. Macrophages derived from neonatal C57BL/6 wild type (ANA-1 macrophages) and C57BL/6 PI3Kγ−/− mice were infected with CFSE-labeled *Leishmania mexicana* promastigotes (7:1 parasites: macrophages) for 1 hour at 33°C/5% CO₂ and analyzed by flow cytometry for quantification of parasitized (CFSE+) macrophages. Data presented are representative of results of 1 of 2 independent experiments with similar results.
Figure 2.5

![Graph showing CFSE staining of L. mexicana promastigotes with two different macrophage genotypes.](image)
Figure 2.6. AS-605240 does not alter expression of *Leishmania* phagocytic receptors by macrophages *in vitro*. C57BL/6 wild type BMDMs were treated with 1.25 μM AS-605240 or DMSO for 3 hours at 37°C/5% CO$_2$ and analyzed for expression of *Leishmania* phagocytic receptor (A, B) complement receptor (CR) 3 (CD11b+/CD18+) and (C) FcγR (CD16/CD32+) by flow cytometry.
Figure 2.6

Complement Receptor (CR) 3

A. [Graph showing FITC-CD18 distribution]

B. [Graph showing PE-CD11b distribution]

C. [Graph showing FcyRII/III distribution with Vehicle and AS-605240 lines]

- Vehicle
- AS-605240
Figure 2.7. PI3Kγ mediates non-specific phagocytosis by macrophages in vitro. AS-605240 inhibits the non-specific uptake of collagen-coated fluorobeads by macrophages. ANA-1 macrophages were treated with 1.25 µM AS-605240 or sterile DMSO (vehicle) and incubated with latex fluorobeads (7:1 beads: macrophages) for 1 hour at 37°C/5% CO₂ and analyzed by flow cytometry for quantification of bead-harboring (FITC+) macrophages. Data presented are representative of results from 1 of 3 independent experiments with similar results.
Figure 2.7

Vehicle

28.3% FITC+
MFI: 3153

AS-605240

3.76% FITC+
MFI: 1831
Figure 2.8. Effect of AS-605240 on phagocytosis of *Leishmania mexicana* by macrophages and neutrophils *in vivo*. Quantification of *Leishmania mexicana* promastigote (A, B) and amastigote (C, D) infected macrophage and neutrophil populations from C57BL/6 mice. Mice were infected with $4 \times 10^6$ CFSE-labeled *Leishmania mexicana* promastigotes (A, B), axenically grown amastigotes (C) or IgG-opsonized axenic amastigotes in the presence or absence of AS-605240 (15 mg/kg; i.p.). Cells were obtained from dorsal pouches by lavage and stained for macrophages (A, C, D) and neutrophils (B) using antibodies against CD68 and GR1, respectively. Panels A-D are representative of results from 2 independent experiments with similar results with a minimum of 4 mice (n=4) per group.
Figure 2. Figure 2.8

Continued
Figure 2.9. *Leishmania mexicana* promastigote entry into macrophages is mediated specifically by Class IB PI3Kγ *in vitro*. Quantification of parasitized macrophages by flow cytometry. ANA-1 macrophages were treated with isoform-selective inhibitors of PI3Kα (10 µM) (C), PI3Kβ (10 µM) (D), PI3Kδ (1 µM) (E), PI3Kγ (1.25 µM) (B), or DMSO vehicle (A). Cells were infected with CFSE-labeled *Leishmania mexicana* promastigotes at 33°C /5% CO₂ for 1 hour (7:1 parasites: cells). Following infection, cells were washed to remove extracellular parasites and analyzed by flow cytometry to quantify parasitized macrophages (CFSE+ cells) in each group. Data presented are representative of results from 1 of 3 independent experiments with similar results.
Figure 2.9

A. Vehicle
49.9% CFSE+

B. AS-605240
15.5% CFSE+

C. PI3-kinase alpha inhibitor 2
66.62% CFSE+

D. TGX-221
62.3% CFSE+

E. IG07114
57.6% CFSE+
CHAPTER 3

Targeting PI3Kγ In The Treatment Of Cutaneous Leishmaniasis Caused By
Leishmania mexicana

3.1 THE USE OF PI3-KINASE INHIBITORS IN INFLAMMATORY DISEASES:

Given the pivotal role for Class IA PI3Kδ and Class IB PI3Kγ in mediating numerous stages and components of the host inflammatory response, much research has been devoted towards to the development of small molecule, pharmacological inhibitors of PI3Kδ and PI3Kγ for therapeutic use in treating various inflammatory-associated diseases. The following chapter will review the current literature regarding the use of isoform-selective PI3-kinase inhibitors in the treatment of various inflammatory-associated disease and will describe our findings using the PI3Kγ isoform-selective kinase inhibitor AS-605240 as a therapeutic for the treatment of cutaneous leishmaniasis caused by Leishmania mexicana.

INFLAMMATORY-MEDIATED RESPIRATORY DISEASES:

Respiratory disease such as allergic asthma and chronic obstructive pulmonary disorder (COPD) represent two inflammatory-mediated diseases where PI3K inhibition may provide useful as an alternative approach to therapy. Allergic asthma and COPD are
characterized by excessive inflammatory cell infiltration, broncho-constriction, and airway/tissue remodeling (233). In most cases, the associated disease pathology can be controlled using corticosteroids, however these treatments are not without side effects and do not prove efficacious in patients with steroid-resistant forms of disease.

Given the important role of PI3Kδ in IgE-mediated mast cell activation, the use of small molecule PI3Kδ isoform-selective PI3K inhibitors represents an attractive strategy for treating allergic disorders including asthma. In murine models of allergic asthma, treatment of mice with the PI3Kδ isoform-selective kinase inhibitor IC87114 was found to limit leukocyte infiltration into the lung tissue, decrease the production of cytokines, chemokines, and inflammatory mediators released into the air space, and to dampen airway mucous production as well as serum IgE levels to limit OVA-induced bronchial constriction in sensitized BALB/c mice (234). Furthermore, PI3Kδ-inhibition by IC87114 was found to prevent airway hyper-responsiveness and constriction of murine tracheal smooth muscle cells induced by IL-13 in vitro (235).

Unlike allergic asthma that is mediated primarily by Th2-type CD4+ T cells and their cytokines, mast cells, eosinophils, and neutrophils, COPD is characterized by the development of abnormal inflammatory responses involving the activation of CD8+ T cells and the production of Th1-associated cytokines and chemokines that promote the recruitment of macrophages, monocytes, and neutrophils into the tissues. Here, these cells contribute to disease progression by producing damaging effector molecules, such as toxic oxygen radicals and proteases that contribute to progressive tissue destruction and fibrosis (233). However, unlike allergic asthma, COPD is often unresponsive towards
standard steroid therapy, highlighting the need for novel anti-inflammatory compounds to treat disease.

Recently, a dual PI3Kδ/PI3Kγ isoform(s)-selective kinase inhibitor, TG100-115, has been shown effective in reducing pathology in allergy-induced asthma as well as smoke- and LPS-induced COPD models of pulmonary inflammation in mice (236). Aerosol-treatment of mice with TG100-115 reduced eosinophilia, IL-13, and mucin accumulation in both allergic- and COPD-models of disease. Importantly, TG100-115 was effective in reducing disease pathology in a steroid-unresponsive model of pulmonary inflammation, suggesting that isoform-selective inhibitors of PI3-kinases may provide a promising alternative for treating non-responsive forms of disease. These findings strongly suggest that the use of isoform-selective pharmacological inhibitors of PI3Kδ and/or PI3Kγ is a viable strategy for treating inflammatory-mediated respiratory diseases in humans.

AUTOIMMUNE DISEASES:

Another area where the therapeutic use of isoform-selective PI3-kinase inhibitors appears promising is in the treatment of inflammatory autoimmune diseases such as systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA). Both SLE and RA are chronic inflammatory diseases/conditions and are associated with the expansion and activation of self-reactive lymphocytes, the recruitment of inflammatory cells, infiltration into the tissues, and development of disease-associated pathology.
SLE is characterized by the expansion of auto-reactive memory T cells that lead to hyperactivation of B lymphocytes and the production of auto-antibodies, ultimately resulting in hypergammaglobulinemia (237). In SLE, auto-reactive B cells specific for DNA produce self-reactive antibodies, which accumulate in the kidneys where they activate complement cascade to promote inflammation and the recruitment of macrophages and T cells into the kidney. These cells contribute to the overall inflammatory state that is responsible for eventual renal failure (237). T cells from both humans and mouse models of SLE exhibit increased levels of phospho-Akt (pAkt), an important effector molecule involved in intracellular signal transduction downstream of PI3K. In mouse models of SLE, the accumulation of activated memory T and B cells has been shown to result from increased lymphocyte activation signals provided by Class I PI3-kinases, further suggesting that targeting PI3Ks using PI3-kinase inhibitors may be an effective strategy for treating disease.

In a mouse model of SLE, intraperitoneal (i.p.) injection of mice with AS-605240 (30 mg/kg) was found to significantly extend the lifespan of experimental mice and was as effective as standard therapy with dexamethasone when administered over a period of 3 months (237). AS-605240-treated mice contained significantly fewer CD4+ T cells in their spleens and lymph nodes as well as fewer CD4+ memory T cells within their kidneys as compared to vehicle-treated control mice. Serum levels of DNA-specific auto-antibodies were reduced in AS-605240-treated mice and were accompanied by decreased proteinuria and diminished glomerulonephritis (237) comparable to that observed in dexamethasone-treated control mice. Furthermore, no increase in
susceptibility to bacterial infections was observed in AS-605240-treated mice. Taken together these findings strongly suggest that selective inhibition of PI3Kγ by AS-605240 may be a viable strategy for treating SLE.

Rheumatoid arthritis is a chronic inflammatory condition characterized by the accumulation inflammatory cells, synovial hyperplasia, and progressive destruction of the cartilage and bone mainly affecting the joints. Development and progression of RA involves both T cells and B cells however the hallmark of disease is the increased expression of chemokines and other chemo-attractants within the inflamed joints which promote the infiltration of leukocytes such as macrophages, neutrophils, mast cells, and T cells, into the tissues (222). Infiltrating leukocytes, as well as fibroblasts contribute to disease pathology by producing destructive matrix metalloproteases and inflammatory mediators responsible for progressive tissue destruction (238).

PI3Kγ is an important mediator of leukocyte chemotaxis, as well as mast cell activation and degranulation, and therefore likely contributes to the progression of inflammatory arthritis by facilitating inflammatory cell infiltration into the tissues and as such, represents an attractive target for alternative strategies to treat disease. In fact, oral administration of the PI3Kγ isoform-selective inhibitor AS-605240 (500 mg/kg) was found to effectively suppress joint inflammation and disease progression without displaying any adverse side effects in experimental models of RA in mice (222).

PI3Kγ can be activated indirectly by tyrosine kinase receptors, and in macrophages, the Class IA PI3K isoform PI3Kδ is primarily responsible for the production and accumulation of PtdIns(3,4,5)P3 in response to activation signals provided
by lipid mediators and/or bacterial products (239). Studies using PI3Kδ isoform-selective kinase inhibitors have demonstrated that pharmacological blockade of PI3Kδ is also effective in reducing inflammatory arthritis in mice (239). Oral administration of the PI3Kδ isoform-selective kinase inhibitor IC87114 (20 mg/kg) effectively reduced inflammation and destruction of cartilage in experimental models of auto-antibody-induced arthritis. Interestingly, IC87114-treatment of PI3Kγ−/− mice resulted in complete resolution of footpad swelling with no sign of cartilage and/or bone destruction, suggesting both PI3Kγ and PI3Kδ contribute to disease progression and that dual PI3Kδ/PI3Kγ-selective kinase inhibitors may be more therapeutically efficacious in the treatment of inflammatory arthritis than single isoform-selective inhibitors of PI3Kδ or PI3Kγ alone (239). In fact, an orally active dual PI3Kδ/PI3Kγ inhibitor, INK1197 (Infinity Pharmaceuticals, Inc., Cambridge, Massachusetts, USA and Intellikine, Inc., La Jolla, California, USA) has undergone preclinical studies in rheumatoid arthritis, allergy, and inflammation and is expected to begin clinical development within the year (2011) (Drug Development and Delivery Technology; July 13, 2010).

**CARDIOVASCULAR DISEASE:**

Atherosclerosis in humans is characterized by the formation of atherosclerotic plaques containing inflammatory cells such as macrophages and T cells in the inner-most layers of the large arteries. Atherosclerosis is a chronic disease that begins with the formation of nascent plaques (fatty streaks) containing significant numbers of lipid-laden macrophages (foam cells) as well as T cells. Over time, these nascent plaques may
disappear or develop into mature plaques (atheromas), which differ in structure and composition as compared to fatty streaks. The core region of mature atherosclerotic plaques contains densely packed foam cells and extracellular lipids surrounded by a collagen-rich fibrous matrix and smooth muscle cell ‘cap’. Macrophages and T cells are especially abundant within the growing plaque however dendritic cells, mast cells, B cells, and NK cells may also be present. Disruption of atherosclerotic plaques may lead to adverse clinical outcomes including thrombosis, myocardial infarction, and/or stroke (240).

Activation of PI3-kinases by modified lipoproteins, cytokines, and chemokines is associated with progression of atherosclerosis. Low-density lipoprotein (LDL) is a growth factor for bone marrow-derived macrophages and is abundant in within atherosclerotic plaques. Studies using broad-spectrum PI3-kinase inhibitors have demonstrated that treatment of macrophages with wortmannin or LY294002 blocks Akt activation and prevents LDL-induced survival and proliferation of these cells in vitro, suggesting that blockade of PI3K signaling pathways may have a beneficial effect in the treatment of atherosclerosis in humans.

PI3Kγ is highly expressed in human and murine atherosclerotic lesions further suggesting that this isoform may be involved in disease progression. PI3Kγ has been shown to be a critical mediator of Akt activation by macrophages in response to several atherosclerotic mediators. Macrophages deficient of p110γ fail to induce activation of Akt in response to LDL and other relevant cytokines/chemokines (241). Furthermore, PI3Kγ-deficient mice develop significantly smaller lesions and are protected against
disease as compared to PI3Kγ$$^{+/+}$$ mice in experimental models of ApoE$$^{-/-}$$-induced atherosclerosis. Treatment of mice with the PI3Kγ isoform-selective inhibitor AS-605240 (10 mg/kg) was also found to reduce plaque development and protect mice against early and advanced lesion development in experimental models of ApoE$$^{-/-}$$ and LDLR$$^{-/-}$$-induced atherosclerotic disease in mice, further suggesting that therapeutic targeting of specific PI3K isoforms may be a viable strategy for treating human disease (242). In fact, TGX100-115, a dual inhibitor of PI3Kδ and PI3Kγ known to suppress vascular leakage and limit lesion development in pre-clinical models, has recently entered Phase I/II clinical trials for the treatment of human cardiovascular disease (243, www.clinicaltrials.gov).

**PI3K INHIBITORS IN CANCER:**

Class I PI3Ks are important regulators of cell growth, survival, metabolism, and motility and as such, are important regulators of cancer progression. Perhaps not surprising is the frequent occurrence of aberrant PI3K signaling observed in human cancers (244) Activation of PI3K/Akt pathways lead to enhanced cell growth and survival through several mechanisms. Activated (phospho-) pAkt inhibits the activity of the pro-apoptotic protein Bcl-2 and activates NF-κB to induce transcription of prosurvival and anti-apoptotic genes. The activity of Akt negatively regulates p53-mediated apoptosis pathways as well as the activity of fork head transcription factors (244, 245).

Genetic mutation of the tumor suppressor protein PTEN is the most frequent alteration of PI3K signaling pathways observed in human cancers. Inactivating mutation
of PTEN results in the accumulation of PtdIns(3,4,5)P$_3$ and constitutive activation of PI3K downstream effector pathways. Although most alterations in the PI3K signaling pathway result from somatic mutation, genetic mutations are associated with some hereditary cancers such as Cowden disease (244).

Given the frequent occurrence of aberrant PI3K signaling observed in many human cancers and the growing evidence supporting constitutive PI3K activation in cancer development and resistance to therapy, the use of pharmacological inhibitors of PI3K pathways would seem a relevant and promising approach for treating human cancers.

Phase I clinical trials using pan-PI3K inhibitors such as GDC-0941 and XL147 have shown some potential anti-tumor activity against human cancers, however these broad-spectrum PI3-kinase inhibitors have been associated adverse side effects such as hyperglycemia and decreased glucose tolerance in animals (244, 246). However, the development of PI3K isoform-selective inhibitors of p110$\alpha$, p110$\beta$, p110$\delta$, and p110$\gamma$ has facilitated a large body of research focused on identifying the role of specific Class I PI3K isoforms in regulating disease progression and identifying potential targets for treatment of human disease.

Mutation in Class IA PI3K$\alpha$ is often seen in human cancers, including breast cancer, colon cancer, endometrial cancers, and glioblastomas (244) and expression of mutant p110$\alpha$ in fibroblasts and mammary epithelial cells leads to growth factor-independent proliferation of cancer cells both in vitro and in vivo. Studies using various Class I PI3K isoform-selective inhibitors have suggested that p110$\alpha$ is a critical regulator
of cell survival pathways in PTEN\(^{+/+}\) cancers and may represent an attractive target for the development of small chemotherapeutics for clinical use (247).

Interestingly, the p110\(\beta\) isoform has been suggested to be a major regulator of cell survival in PTEN-mutant cancer cell lines (248). PI3K\(\beta\) activity has been shown to play an important role in signaling by prostate cancer cells. Inhibition of PI3K\(\beta\) activity by short hairpin RNA (shRNA) and/or by treatment with small molecule PI3K\(\beta\) isoform-selective inhibitor TGX-221 reduced pAkt levels and limited cell growth of PTEN-mutant tumor cells both \textit{in vitro} and \textit{in vivo} (249). Similarly, specific inhibition of p110\(\beta\) was found to negatively effect cell growth and survival of several breast cancer cell lines \textit{in vitro} (250). Taken together, these studies strongly suggest that while specific inhibition of p110\(\alpha\) may be effective in the treatment of human cancers associated with functional PTEN activity, the Class IA p110\(\beta\) isoform may provide a more useful target than PI3K\(\alpha\) in the treatment of cancers associated with functional loss of PTEN activity.

Chronic myeloid leukemia is a disorder of the hematopoietic system and is characterized by excess accumulation of immature and mature myeloid cells. CML is caused by a mutation in two genes (Bcl and Abl; Philadelphia chromosome) resulting in the expression of a constitutively active p210 fusion protein with tyrosine kinase activity and activation of several signal transduction pathways including PI3K-Akt pathways (251). Transfection of 32D myeloid progenitor cells with mutant BCL-ABL results in growth factor-independent cell growth and resistance against cytotoxic drug-induced apoptosis \textit{in vitro}. In \textit{vitro} studies have demonstrated the selective up-regulation in expression as well as lipid and protein kinase activity of the Class IB PI3K\(\gamma\) isoform in
response to mutant BCR-ABL in several BCR-ABL-transfected cell lines. Furthermore, transfection of cell lines with double negative mutant of PI3Kγ was found to significantly reduce cell proliferation and drug-induced apoptosis in vitro.

In contrast, the Class IA PI3Kδ isoform appears to play a predominant role in PI3K signaling in acute myeloid leukemias. Treatment with the PI3Kδ isoform-selective inhibitor IC87114 inhibited cell proliferation and survival of both AML primary cells and human AML-derived cell lines in vitro (252, 253). Recently, the PI3Kδ isoform-selective kinase inhibitor CAL-101 was found to induce cell death of human chronic lymphocytic leukemia (CLL) cells and to limit the production of inflammatory cytokines associated with disease progression by NK and T cells (254). In fact, CAL-101 has been shown to inhibit Akt activation and to induce cell death against several leukemia, lymphoma, and myeloma cell lines and has shown promising results in Phase I clinical trials of refractory non-Hodgkin’s lymphoma and chronic lymphocytic leukemia.

3.2 RESULTS:

*Leishmania* spp. are obligate intracellular pathogens of mammals, and parasite survival and replication within the host requires that parasites gain access to host cells where they may be protected from destruction by the host immune response. *Leishmania* spp. lack the necessary machinery required for active penetration of host cells and therefore are limited to infecting professional phagocytes such as neutrophils and macrophages, with the latter acting as the definitive host cell for parasite survival and replication. Resistant to the acidic pH and hydrolytic environment within the
phagolysosome, parasites undergo unrestricted replication ultimately leading to host cell lysis and the release of infectious parasites.

Studies using broad-spectrum PI3-kinase inhibitors such as wortmannin and LY294002 have shown that Class I PI3Ks are involved in phagocytosis and mediating the entry of parasites such as Trypanosoma cruzi into host cells (218-220), however the precise role of distinct PI3K isoforms remains unclear. Using pharmacologic and genetic tools, we identified an important role for the Class IB PI3K_γ isoform in mediating the entry of Leishmania parasites into macrophages and neutrophils. Both genetic deficiency, or pharmacologic inhibition using the p110γ isoform-selective kinase inhibitor, AS-605240, of PI3K_γ was found to significantly reduce the entry of Leishmania mexicana parasites into macrophages and neutrophils both in vitro and in vivo. These findings suggest that PI3K_γ mediates susceptibility to Leishmania infection by facilitating parasite entry into host cells during infection and suggest that specific targeting of PI3K_γ using isoform-selective kinase inhibitors may be a viable strategy for treating cutaneous leishmaniasis caused by Leishmania mexicana.

Because AS-605240 displayed no cytotoxic effects against host cells in vitro (Figure 3.1 and Figure 3.2) and was effective in reducing parasite entry into macrophages and neutrophils both in vitro and in vivo (See Chapter 2), we sought to determine whether AS-605240 might be used as a therapeutic to treat cutaneous leishmaniasis caused by Leishmania mexicana. Using a low dose model of cutaneous Leishmania mexicana infection that closely mimics natural transmission in both the location and the number of parasites, we examine the effect of daily treatments with AS-605240 on disease
progression in C57BL/6 mice. C57BL/6 wild type mice were infected with 1000 *Leishmania mexicana* promastigotes into the left ear and disease progression was monitored by measuring the increase in thickness of the infected ear to that of the uninfected ear at weekly intervals.

Two weeks post-infection, mice began receiving 2X daily injections (i.p.) with AS-605240 (15 mg/kg) or saline vehicle for a period of 4 weeks. C57BL/6 mice treated with AS-605240 developed significantly smaller lesions as compared to untreated (saline) controls (Figure 3.3 A and Figure 3.4). Histopathological analysis of the infected lesions of control mice by routine hematoxylin and eosin staining revealed heavy inflammatory cell infiltrates consisting of heavily parasitized macrophages, neutrophils, and eosinophils (Figure 3.5 B). In contrast, lesions from *Leishmania mexicana*-infected C57BL/6 mice treated with AS-605240 (Figure 3.5 A) contained significantly fewer macrophages (Figure 3.6 A) and neutrophils (Figure 3.6 B) within the lesions as compared to control mice treated with saline.

Determination of parasite burdens within the infected lesions by limiting dilution analysis revealed that in addition to developing smaller lesions, *Leishmania mexicana*-infected C57BL/6 mice treated with AS-605240 harbored significantly lower parasite burdens as compared to saline-treated controls. (Figure 3.3 B).

Eight weeks post-infection, the draining lymph node cells from AS-605240- and saline-treated mice were isolated and stimulated *in vitro* with *Leishmania mexicana* antigen (LmAg) and the levels of Th1-associated cytokines IL-12p70 and IFN-γ and Th2-associated cytokines IL-4 and IL-10 were determined by ELISA (Figure 3.7 A-D).
Draining lymph node cells obtained from AS-605240-treated mice and stimulated with LmAg produced less IL-4 (Figure 3.7 C) and IL-10 (Figure 3.7 D) as compared to untreated (saline) controls, with the latter being statistically significant. Interestingly, no increase in the levels of the protective Th1 cytokines IL-12 and IFN-γ (Figure 3.7 A, B) were observed in AS-605240-treated draining lymph node cells despite the increased resistance to Leishmania mexicana infection observed in these mice. Furthermore, AS-605240 was not found to have any effect on Th1 or Th2 cytokine production by naive T cells stimulated in vitro with anti-CD3/anti-CD28 antibodies suggesting that the suppression of immunosuppressive Th2 cytokines is an indirect effect of AS-605240 on T cells (data not shown).

In order to determine whether the decreased levels of IL-10 detected in the draining lymph nodes of AS-605240-treated mice was a result of impaired expansion, tissue homing, and/or effector function of CD4+ FoxP3+ regulatory T cells, we analyzed the effect of AS-605240-treatment on recruitment and IL-10 production by CD4+ FoxP3+ regulatory T cells using C57BL/6 FoxP3-EGFP knock-in mice. AS-605240-treatment of Leishmania mexicana-infected C57BL/6 FoxP3-EGFP mice significantly impaired the recruitment of CD4+ FoxP3+ regulatory T cells to the site of infection (Figure 3.8 A). No differences were observed in CD4+ FoxP3+ Treg populations within the draining lymph nodes of AS-605240- and vehicle-treated mice (Figure 3.8 B). Furthermore, analysis IL-10 production by ELISA (A) and flow cytometry (B) revealed no significant change in cytokine production by AS-605240-treated and control CD4+ FoxP3+ regulatory T cells in vitro (Figure 3.9). Naïve C57BL/6 wild type and PI3Kγ−/−
mice were found to contain comparable proportions of CD4+ FoxP3+ regulatory T cells within their spleens (Figure 3.10 A) and lymph nodes (Figure 3.10 B) indicating that PI3Kγ is not required for the generation of these cells in vivo.

Because AS-605240 treatment effectively limited lesion development, decreased parasite burdens at the site of infection, and reduced the production of the immunosuppressive cytokine IL-10, we were interested in comparing the therapeutic efficacy of AS-605240 to that of standard chemotherapy the with pentavalent antimony Pentostam® sodium stibogluconate. C57BL/6 were infected with low dose Leishmania mexicana as described previously, and beginning two weeks post-infection, received 2X daily treatment with AS-605240 (15 mg/kg), sodium stibogluconate (SSG) (15 mg/kg) or saline vehicle for a period of 14 days. As expected, control mice treated only with saline developed progressive, ulcerating lesions. Five weeks after the cessation of treatment, Leishmania mexicana-infected mice from each group were euthanized and parasite burdens within the infected ear determined by limiting dilution analysis. As expected, saline-treated control mice harbored significant numbers of parasites within their lesions. In contrast, the infected ears from mice treated with the PI3Kγ inhibitor, AS-605240, or sodium stibogluconate contained significantly fewer parasites (nearly 4-5 log less) as compared to untreated (saline) controls (Figure 3.11 A).

We also determined wither treatment with AS-605240 was effective in reducing disease progression and pathology when administered late after infection, following onset of clinical disease. C57BL/6 wild-type mice were infected with Leishmania mexicana as described previously, however treatment with either AS-605240 or sodium
stibogluconate did not commence until six weeks-post infection, after the development of visible, ulcerating lesions. Groups of mice were treated (i.p.) 2X daily with AS-605240 (15 mg/kg), sodium stibogluconate (15 mg/kg), or saline vehicle for a period of 14 days. Two weeks following the cessation of treatment (10 weeks post-infection), infected mice from each group were euthanized and parasite burdens determined by limiting dilution analysis. The ears from *Leishmania mexicana*-infected mice treated with sodium stibogluconate harbored ~10X fewer parasites as compared to saline controls (Figure 3.11 B).

Histopathological analysis of the infected tissues obtained from *Leishmania mexicana*-infected C57BL/6 saline-treated control mice revealed significant tissue inflammation and extensive inflammatory infiltration comprised of heavily parasitized macrophages, neutrophils, and eosinophils (Figure 3.12 A). In contrast, the infected ears of mice treated with AS-605240 exhibited a substantial reduction in tissue inflammation, comparable to that observed in Pentostam® sodium stibogluconate-treated mice (Figure 3.12 B, C). Furthermore, the infected lesions from AS-605240-treated mice harbored significantly fewer parasites within the infected lesions as compared to untreated (saline) controls. More importantly however, similar parasite burdens were observed within the infected lesions of AS-605240- and Pentostam® sodium stibogluconate- treated mice (Figure 3.11 B).

Finally, we determined the efficacy of AS-605240 to limit disease progression when administered in conjunction with Pentostam® sodium stibogluconate. C57BL/6 WT mice were infected with low dose *Leishmania mexicana*, as described previously,
and received 2X daily treatment with saline vehicle, AS-605240 (15 mg/kg), sodium stibogluconate (15 mg/kg), or a combination AS-605240 + SSG for 2 weeks. *Leishmania mexicana*-infected mice treated with a combination therapy of AS-605240 and Pentsotam® sodium stibogluconate developed smaller lesions harboring fewer parasites than mice treated with either AS-605240 or Pentostam® sodium stibogluconate alone when administered either early or late after infection (Figure 3.11 A, B).

### 3.3 DISCUSSION:

The Class I PI3-kinases PI3Kδ and PI3Kγ exhibit preferential expression by cells of the immune system and play significant roles in controlling numerous components and stages of the host inflammatory response. Given the pivotal role for PI3Kδ and PI3Kγ in inflammation, significant research has been directed towards understanding the specific function of distinct PI3K isoforms in health and disease. The availability of genetically modified mice, as well as the development of isoform-selective inhibitors of specific PI3K isoforms has greatly augmented our understanding of the role of distinct PI3K isoforms and has prompted the development of numerous small-molecule pharmacological PI3-kinase inhibitors for preclinical and clinical use.

AS-605240 (5-quinoxalin-6-ylmethylene-thiazolidine-2,4-dione) is a small-molecule, orally active PI3Kγ isoform-selective kinase inhibitor that effectively out-competes ATP for its binding pocket, rendering the kinase inactive (222). AS-605240 was found to reduce neutrophil recruitment in response to RANTES (CCL5) in a mouse model of peritonitis with a half-maximal efficacious dose (ED₅₀) value of 9.1 mg/kg.
(222) and oral administration of AS-605240 (50 mg/kg) was effective in reducing footpad swelling, synovial inflammation, and cartilage erosion in experimental models of collagen-induced arthritis in mice (222).

Because PI3Kγ inhibition by AS-605240 was found to significantly impair the entry of *Leishmania* parasites into macrophages and neutrophils both *in vitro* and *in vivo*, we examined whether the use of AS-605240 might be effective in reducing disease progression of cutaneous leishmaniasis when used as a therapeutic to treat disease. Using a low dose model of *Leishmania mexicana* infection that closely mimics natural infection, we show that AS-605240 is therapeutically efficacious when used as a chemotherapeutic to treat cutaneous *Leishmania mexicana* infection.

*Leishmania mexicana*-infected C57BL/6 mice treated with AS-605240 (15 mg/kg) developed significantly smaller lesions harboring fewer parasites as compared to ‘untreated’ (saline) controls. *Leishmania mexicana*-infected control mice developed progressive lesions showing ulceration and necrosis and harbored significant numbers of parasitized macrophages, neutrophils, and eosinophils within their lesions. The lesions of AS-605240-treated mice, however, contained significantly fewer macrophages and neutrophils within the infected lesions. This is perhaps not surprising, as PI3Kγ is a well-known mediator of macrophage and neutrophil migration both *in vitro* and *in vivo*. In macrophages and neutrophils, the Class IB PI3Kγ isoform is a critical mediator of actin polymerization and cytoskeletal reorganization in response to chemotactic stimuli and macrophages and neutrophils from PI3Kγ−/− mice exhibit impaired migration in response to numerous chemokines and other GPCR-associated inflammatory stimuli (181, 182,
197, 247). The reduced number of macrophages and neutrophils observed in the lesions of AS-605240-treated mice likely contributes to the enhanced resistance observed in these mice.

The Th2-associated cytokines IL-4 and IL-10 are known susceptibility factors in cutaneous leishmaniasis and are associated with disease progression and parasite persistence in experimental models of murine and human cutaneous leishmaniasis (123). IL-4 production by activated CD4+ T cells contributes to the suppression of IL-12-driven expansion of Th1-type CD4+ T cells and the production of IFN-γ required to activate macrophages to kill intracellular parasites. IL-10 is essential for persistence of parasites and contributes to susceptibility to infection by preventing the activation of antimicrobial effector functions of macrophages to promote parasite survival within the host (138-141). C57BL/6 mice deficient of IL-10 (IL-10−/−) produce more IFN-γ, nitric oxide, and completely resolve *Leishmania mexicana* infection (139).

The resistant phenotype observed in IL-10−/− mice is dependent upon the production of IL-12 by macrophages, neutrophils, and dendritic cells. The treatment of IL-10−/− mice with anti-IL12p40 monoclonal antibodies completely abrogated the healing phenotype observed in *Leishmania mexicana* IL-10−/− mice, highlighting the importance of IL-12 in driving the expansion cell-mediated Th1-type immune responses capable of controlling parasite survival and replication within the host (139). Consistent with the role of an IL-12-driven protective Th1-type cell mediated immune response in protection against *Leishmania* infection, mice lacking IFN-γ, iNOS, or STAT4 are extremely
susceptible to *Leishmania mexicana* infection and develop uncontrolled progressive lesions harboring large numbers of parasites as compared to wild type mice (255).

Analysis of Th1/Th2 cytokine production within the draining lymph nodes of AS-605240-treated and control mice revealed a significant decrease in the production of IL-10 by draining lymph node cells of AS-605240-treated mice. Interestingly, AS-605240-treated mice exhibited an increase in resistance to *Leishmania mexicana* infection despite producing comparable levels of the Th1-associated cytokines IL-12 and IFN-γ. AS-605240 did not impair the production of either Th1 or Th2 cytokines by naïve CD4+ T cells stimulated with anti-CD3/anti-CD28 polyclonal antibodies *in vitro*, suggesting that AS-605240 does not have a direct effect on cytokine production by T cells.

Regulatory T cells suppress immune responses by producing cytokines, namely IL-10, that limit inflammatory responses and contribute to the development of protective immunity in cutaneous leishmaniasis by regulating the maintenance of low numbers of parasites ($10^2-10^4$; enough to maintain the survival of *Leishmania*-specific memory T cells) within the skin and draining lymph nodes of healed patients. A study by Belkaid *et al.* 2002 demonstrated that CD4+ FoxP3+ regulatory T cells contribute to pathogenesis of cutaneous leishmaniasis by suppressing the activity of CD4+ effector T cells via IL-10-dependent and –independent mechanisms (142). In experimental models of *Leishmania major* infection in susceptible and resistant BALB/c and C57BL/6 mice, respectively, IL-10 production by CD4+ CD25+ FoxP3+ regulatory T cells contributes to the persistence of parasites within the host and depletion of these cells renders susceptible BALB/c mice...
resistant to infection, further supporting a non-protective role for regulatory T cells in cutaneous leishmaniasis.

A recent study by Liu et al. 2009 reported that PI3Kδ, a Class IA PI3K isoform also expressed by leukocytes that regulates actin polymerization and cytoskeletal reorganization together with PI3Kγ, controls susceptibility to *Leishmania major* (201). C57BL/6 and BALB/c mice expressing an inactivating knock-in mutation of PI3Kδ (p110δ^D910A^) exhibit increased resistance to *Leishmania major*, as evidenced by the development of smaller lesions harboring lower parasite burdens, as compared to wild type mice regardless of genetic background (201).

The protective phenotype observed by PI3Kδ mutant mice was characterized by a decrease in cell proliferation and production of effector cytokines IFN-γ, IL-10 and TNF by draining lymph node cells. Interestingly PI3Kδ- ‘deficiency’, unlike PI3Kγ blockade by AS-605240, impaired cytokine production by naïve CD4+ T cells stimulated by anti-CD3/anti-CD28 polyclonal antibodies *in vitro*, further supporting that PI3Kδ, rather than PI3Kγ, is primarily responsible for regulating cytokine production by CD4+ T cells.

Further analysis of CD4+ T cell populations in *Leishmania major*-infected C57BL/6 WT and PI3Kδ- mutant mice suggested that the increased resistance observed in the absence of PI3Kδ was a result of impaired expansion, tissue homing, and suppressive capacity (IL-10 production) of CD4+CD25+FoxP3+ regulatory T cells (201). PI3Kδ- mutant mice contained significantly fewer CD4+ CD25+ FoxP3+ regulatory T cells within their spleens and draining lymph nodes as compared to wild type mice, suggesting that PI3Kδ is important for the expansion of Tregs in response to *Leishmania*
major infection. PI3Kδ was also required for proper trafficking of CD4+ CD25+ FoxP3+ Tregs to the site of infection, as evidenced by the reduced numbers of these cells within the lesions of PI3Kδ- mutant mice as compared to PI3Kδ+/+ WT controls. Furthermore, PI3Kδ-‘deficiency’ significantly impaired the ability of these cells to produce IL-10. Adoptive transfer studies confirmed that the impaired expansion, tissue homing, and effector function of PI3Kδ-‘deficient’ Tregs was responsible for the enhanced resistant phenotype observed in PI3Kδ- mutant mice.

For this reason, we examined if the increased resistance observed in PI3Kγ−/− and AS-605240-treated WT mice to *Leishmania mexicana* infection was due to impairment in the development, expansion, tissue homing, and/or effector function of CD4+FoxP3+ regulatory T cells. Using C57BL/6 FoxP3-EGFP transgenic mice, we examined the effect of AS-605240-treatment on CD4+ FoxP3+ regulatory T cells in cutaneous *Leishmania mexicana* infection. AS-605240-treated mice contained significantly fewer CD4+ FoxP3+ regulatory T cells within their lesions as compared to vehicle-treated controls. No significant differences were observed in Treg populations within the draining lymph nodes of AS-605240 and control mice, suggesting that PI3Kγ inhibition by AS-605240 does not have an effect on the expansion of these cells in vivo but does impair the recruitment of these cells to the site of *Leishmania mexicana* infection. C57BL/6 PI3Kγ−/− and wild type mice contained comparable proportions of CD4+ FoxP3+ regulatory T cells within their spleens and lymph nodes. Furthermore, AS-605240-treatment was not found to have any effect on IL-10 production (either secreted IL-10 or intracellular IL-10) by naïve CD4+ FoxP3+ T cells in vitro. Taken together, these findings strongly argue
against a major role for the Class IB PI3Kγ in the development, expansion, or effector function of regulatory T cells in vivo. However, PI3Kγ deficiency or inhibition by AS-605240 results in impaired recruitment of cells to the site of infection. Taken together, these findings suggest that PI3Kγ does not regulate development, expansion, or IL-10 production by regulatory T cells to mediate susceptibility to Leishmania mexicana, but rather contributes to the pathogenesis of cutaneous leishmaniasis by mediating the recruitment of these cells to the site of Leishmania mexicana infection. These findings are consistent with findings by Anderson et al. 2007 reporting that CD4+ CD25− FoxP3+ T cells are the source of IL-10-mediated immune suppression in chronic cutaneous leishmaniasis (256).

Treatment of Leishmania mexicana-infected C57BL/6 mice with AS-605240 was found to be as effective as standard anti-leishmanial therapy with Pentostam® sodium stibogluconate when administered either early before the development of visible lesions, or late after the onset of clinical disease. Eight weeks post-infection, AS-605240- and Pentostam® sodium stibogluconate-treated mice harbored significantly lower parasite burdens as compared to vehicle-treated controls. Interestingly, similar parasite burdens were detected in the lesions of AS-605240- and Pentostam® sodium stibogluconate-treated mice, indicating that the PI3Kγ isoform-selective inhibitor, AS-605240, is therapeutically as effective as standard anti-leishmanial therapy at reducing parasite growth. As expected, histopathological analysis of infected lesions from AS-605240- and Pentostam® sodium stibogluconate-treated mice exhibited a substantial reduction in inflammatory cell infiltration in the tissues as compared to vehicle treated controls, also
reflecting the diminished pathology (lesion development/ulceration/necrosis) observed visually.

Combination therapy with both AS-605240 and Pentostam® sodium stibogluconate resulted in an even greater reduction in parasite burdens and a similar decrease in associated disease pathology suggesting that a combination therapy involving both a PI3Kγ isoform-selective kinase inhibitor along with standard anti-leishmanial chemotherapy may be a more efficacious treatment strategy than standard antimonial therapy alone. Furthermore, it may not be unreasonable to suggest that treatment strategies designed at targeting host pathways involved in disease progression rather than the parasite directly may circumvent problems associated with the development of parasite resistance against treatment.

### 3.4 MATERIALS AND METHODS:

**ANIMALS:**

Eight-10 –week old C57BL/6 mice (female) were purchased from Harlan® (Harlan®, Indianapolis, Indiana, USA). PI3Kγ- deficient C57BL/6 mice (back crossed 10 times) were originally a generous gift of Dr. Bao Lu. C57BL/6 EGFP-FoxP3 reporter mice were a generous gift of Dr. Arlene Sharpe. C57BL/6 wild type (WT) and C57BL/6 PI3Kγ<sup>−/−</sup>, and C57BL/6 EGFP-FoxP3 mice were maintained in a pathogen free animal facility at The Ohio State University in accordance with National and Institutional guidelines.
PARASITES:

*Leishmania mexicana* (MNYC/BZ/62/M379) was maintained by serial passage of amastigotes inoculated subcutaneously (s.c.) into the shaven rumps of C57BL/6 mice. Amastigotes were obtained from the infected lesions and grown *in vitro* in M199 media (GIBCO® Invitrogen™ Carlsbad, California, USA) supplemented with 10% FBS (Atlanta® Biologicals, Lawrenceville, Georgia, USA) and 1% Penicillin/Streptomycin (GIBCO® Invitrogen™ Carlsbad, California, USA) at 26°C without CO₂ to generate *Leishmania mexicana* promastigotes for both *in vitro* and *in vivo* studies.

INFECTION PROTOCOL:

Metacyclic promastigotes for infections were obtained by negative selection by agglutination with peanut lectin. Early passage *Leishmania mexicana* promastigotes were washed in Hanks Balanced Salt Solution (HBSS, 1X) (GIBCO® Invitrogen™ Carlsbad, California, USA) 3 times by centrifugation at 3000 RPM for 5 minutes at 4°C and suspended at a concentration of 1.5 x 10⁸ parasites/mL. Lectin from *Arachis Hypogaea* (peanut) (Sigma® Aldrich, St. Louis, Missouri, USA) was added to the parasite suspension (75 µg/mL; final concentration) and incubated at room temperature for 30 minutes. Peanut lectin-agglutinated parasites were centrifuged at 200 RCF for 5 minutes at 4°C and the metacyclic (non-agglutinated) promastigotes were collected from the supernatant and re-suspended in RPMI 1640 cell culture media (GIBCO® Invitrogen™ Carlsbad, California, USA) supplemented with 10% FBS and 1% Penicillin/Streptomycin (GIBCO® Invitrogen™ Carlsbad, California, USA). Experimental animals were infected
intradermal (i.d.) into the left ear dermis with $1 \times 10^3$ PNA-selected *Leishmania mexicana* promastigotes.

**ANTIBODIES AND REAGENTS:**

PE-conjugated anti-CD68, PE-conjugated anti-F4/80, and PE-conjugated anti-GR1 (Biolegend®, San Diego, California, USA) antibodies were used to quantify infected macrophages and neutrophils by flow cytometry. FITC-conjugated anti-CD4 and Alexa Fluor® 697-conjugated anti-FoxP3 (Biolegend®, San Diego, California, USA) antibodies were used to quantify CD4+ FoxP3+ regulatory T cells. BMDMs were stained in 1:20 dilution of Giemsa (modified) stain (Sigma Aldrich™, Saint Louis, Missouri, USA) at room temperature for 20 minutes.

**TREATMENT PROTOCOL:**

AS-605240 (15 mg/kg), Pentostam® sodium stibogluconate (15 mg/kg) or 1X sterile saline (0.9% NaCl) was administered 2X daily by intraperitoneal (i.p.) injection for 14-28 days. For combination treatment, mice received 2X daily treatment with AS-605240 (15 mg/kg) + Pentostam® sodium stibogluconate (15 mg/kg) for 14-28 days. Treatment began either 2 weeks or 6 weeks post-infection. Mice were sacrificed 2 weeks after the cessation of treatment for analysis of parasite burdens, histopathology, and cytokine profiles of infected lesions and draining lymph node cells, respectively.
LESION DEVELOPMENT:
Disease progression was monitored at weekly intervals by measuring the diameter of the infected ear minus the diameter of the uninfected ear. The ears were measured using a Mitutoyo Pocket Thickness Gage (Mitutoyo American Corporation, Aurora, Illinois, USA). Data is presented as mean lesion size in millimeters +/- standard error from 3 independent experiments (n= 15 mice per group).

PARASITE BURDENS:
Parasite burdens within the infected ears were determined by limiting dilution analysis. The infected ears were removed, separated, and passed through a 70 µm nylon cell strainer (BD Falcon™, Bedford, Massachusetts, USA). Cells were washed in PBS and serially diluted (1/10 dilutions) across a Costar® 96-well flat bottom tissue culture plate (Corning Incorporated, Corning, New York, USA) in M199 cell culture media in duplicate and incubated at 26°C without CO₂ for 5-10 days. At this time the greatest dilution yielding viable parasites was recorded for each ear. Data presented as mean parasite dilution +/- standard error from 2-3 independent experiments (n = 10-15 mice per group).

T CELL PROLIFERATION:
The draining lymph nodes of *Leishmania mexicana*-infected mice were obtained 8 weeks post infection. Draining lymph node cells (3 x 10⁶) were added to the wells of a Costar® 96-well flat bottom tissue culture plate and stimulated with freeze-thawed *Leishmania*
mexicana antigen (LmAg) (40 µg/mL; final concentration) or supplemented RPMI 1640 cell culture media as a negative control. Cells were incubated at 37°C/5% CO₂ for 72 hours at which time the supernatants were collected from parallel cultures for ELISA quantification of IL-10, IL-4, IL-12p70, and IFN-γ cytokine production.

**CYTOKINE ELISA:**
IL-10, IL-4, IL-12p70, and IFN-γ levels were determined by sandwich ELISA method using recombinant cytokines as standards (BD Pharmingen™, San Diego, California, USA) and high binding 96-well Nunc Maxisorp™ ELISA plates (Nalge Nunc International, Rochester, New York, USA). The antibody clones used are as follows: IL-12 clone C18.2 (capture; Biolegend®, San Diego, California, USA) and C17.8 (IL-12p40; detection; BD Pharmingen™, San Diego, California, USA), IL-4 clone 11b11 (capture) and BVD6-24G2 (detection) (BD Pharmingen™, San Diego, California, USA), IL-10 clone JES5-16E3 (capture) and JES5-2A5 (detection) (BD Pharmingen™, San Diego, California, USA), and IFN-γ clone R46A2 (capture; BD Pharmingen™, San Diego, California, USA) and XM91.2 (detection; Biolegend®, San Diego, California, USA). ELISA plates were read at 405 nm using a SpectraMax M3 microplate reader (Molecular Devices, Sunnyvale, California, USA). Data is presented as mean cytokine concentration (pg/mL) +/- standard error.
HISTOPATHOLOGY:

The infected ears of *Leishmania mexicana*-infected mice were removed, separated, and fixed in 10% buffered formalin for 7 days. The lesions were embedded in paraffin and 4-8 µM sections were cut. The tissues were mounted on glass slides, hydrated, and stained by routine hematoxylin and eosin staining, for visualization by standard light microscopy.

CELL CULTURE AND ISOLATION:

Bone marrow-derived macrophages (BMDMs) were obtained from bone marrow-derived (BMD) cells isolated from the femurs and tibias of C57BL/6 wild type mice. BMD cells were cultured in RPMI 1640 cell culture media (GIBCO® Invitrogen™, Carlsbad, California, USA) supplemented with 10% FBS and 1% Penicillin/Streptomycin, and 20% supernatants from *in vitro*-cultured L929 cells at 37°C/5% CO₂ for 7-10 days in vented 75 cm² CELLSTAR® cell culture flasks (Greiner Bio-One, Frickenhausen, Germany).

TRANSMISSION ELECTRON MICROSCOPY:

All steps were carried out in microcentrifuge tubes, centrifuging and re-suspending between each processing step. Parasite and cells were washed with 1X PBS and fixed in 3% buffered (0.2M sodium cacodylate pH=7.4) glutaraldehyde solution for 7 days. The samples were rinsed 2X in buffer (10 minutes each), followed by 1% osmium tetroxide in sym-collidine buffer (pH=7.6) for 1 hour followed by two 10 minute buffer rinses and then enbloc stained with 4% uranyl acetate (aq) for 1 hour. Cells were then dehydrated with a graded ethanol series (30%, 50%, 70%, 90%, 95%, 3 x 100%) for 10 minutes at
each step. Transitional solvent used was acetone (3 x 10 minutes). Cells were then infiltrated with 50% Spurr’s epoxy resin in acetone overnight. Two 100% changes of 100% resin for 1 hour each were performed. Resin block polymerization was carried out at 65°C for 24 hours. Thin sections (80 nm) were obtained using a Leica UCT ultramicrotome, collected on 200 mesh copper grids. Sections were post-stained with Reynolds lead citrate for 3 minutes. Sections were then examined on a JEOL JEM-1400 TEM operating at 80 KV. Digital images were obtained using an Olympus-SIS Veleta digital camera.

**STATISTICAL SIGNIFICANCE:**

Statistical significance of values obtained for lesion sizes and cytokine concentrations were determined by an unpaired students’ $t$ test. For limiting dilution analysis of parasite burdens, statistical significance was determined by a Mann-Whitney $U$ prime test.
Figure 3.1. AS-605240 does not display cytotoxic effects on macrophages in vitro. C57BL/6 BMDMs were plated on glass cover slips and allowed to adhere overnight. Cells were treated with 1.25 µM AS-605240 (B) or DMSO (A) and incubated at 37°C/5% CO₂ for 0-3 hours. Following incubation, cells were washed with PBS to remove excess drug and media, fixed in 100% methanol, stained with Giemsa, and mounted on glass slides for visualization by standard light microscopy. Data presented are representative images (40X) obtained at 30 minutes and 180 minutes post-treatment.
Figure 3. 1

A. **Vehicle**

B. **AS-605240**
Figure 3.2. Electron microscopy analysis of effect of AS-605240 on macrophages in vitro. C57BL/6 BMDMs were plated on glass cover slips and allowed to adhere overnight. Cells were treated with 1.25 µM AS-605240 (B) or DMSO (A), infected with *Leishmania mexicana* axenic amastigotes, and incubated at 33°C/5% CO₂ for 3 hours. Following incubation, cells were washed with PBS to remove excess drug and media, fixed in 3% buffered glutaraldehyde solution for 7 days, and processed and stained for visualization by (transmission) electron microscopy.
Figure 3.2

A. Vehicle

B. AS-605240
Figure 3.3. Effect of AS-605240 on disease progression and pathogenesis of cutaneous *Leishmania mexicana* infection. (A) Disease progression as monitored by measuring the thickness of infected ears using a dial-gauge micrometer at weekly intervals. Treatment began 2 weeks post-infection and continued for 4 weeks. Mice were treated 2X daily with AS-605240 (15 mg/kg) or 1X sterile saline (vehicle). Data shown are from 3 independent experiments with similar results and are expressed as mean lesion size (mm) +/- standard error (n= 15 mice) for each group. * indicates p< 0.05 as determined by an unpaired Students’ *t* test. (B) Parasite burdens at week 8 post-infection as determined by limiting dilution analysis. Cells from infected ears were serially diluted across a 96 well microtiter plate in duplicate and incubated at 26°C without CO₂ for 7-10 days when the greatest dilution yielding viable parasites was recorded for each ear. Data presented as mean log parasite dilution +/- standard error from 3 independent experiments (n= 15 mice per group) with similar results. * indicates p< 0.05 as determined by a Mann Whitney *U* prime test.
Figure 3. 3

**A. Lesion Diameter**

- **Mean Lesion Size (mm)**
  - Vehicle
  - AS-605240

- Weeks Post-Infection:
  - 1, 2, 3, 4, 5, 6, 7, 8

- *p < 0.05

**B. Parasite Burdens**

- **Log Parasite Dilution**
  - Vehicle
  - AS-605240

- *p < 0.05
Figure 3.4. Effect of AS-605240 on disease progression and pathogenesis of cutaneous *Leishmania mexicana* infection. Mice were infected with 1000 stationary phase *Leishmania mexicana* promastigotes into the left ear dermis. Mice were treated 2X daily (i.p.) with AS-605240 (15 mg/kg) (B) or 1X sterile saline (vehicle) (A). Treatment began 2 weeks post-infection and continued for 4 weeks. Mice were sacrificed at 8 weeks post-infection. Images shown are representative of results from 1 of 3 independent experiments with similar results (*n* = 15 mice per group).
Figure 3.4

A. Lesions from Vehicle-Treated Control Mice

B. Lesions from AS-605240-Treated Experimental Mice
Figure 3.5. Histopathological analysis of *Leishmania mexicana*-infected ear lesions from AS-605240- and vehicle-treated C57BL/6 mice. Mice were infected with 1000 stationary phase *Leishmania mexicana* promastigotes into the left ear dermis. Mice were treated 2X daily (i.p.) with AS-605240 (15 mg/kg) or 1X sterile saline (vehicle) beginning 2 weeks post-infection and continuing for 4 weeks. Mice were sacrificed at 8 weeks post-infection. Data shown are representative hematoxylin and eosin stained histology images of *Leishmania mexicana*-infected lesions from (A) vehicle- and (B) AS-605240-treated mice from 1 of 3 independent experiments with similar results.
Figure 3.5

A. Vehicle

B. AS-605240
Figure 3.6. Analysis of macrophage and neutrophil populations within the lesions of *Leishmania mexicana*-infected AS-605240-treated and control mice. C57BL/6 mice were infected with 1000 *Leishmania mexicana* promastigotes and treated (i.p.) with AS-605240 (15 mg/kg) or vehicle for 4 weeks. Six- and 9 weeks post-infection, mice were sacrificed and the infected ears removed, separated, and cells isolated for flow cytometric analysis of macrophage and neutrophil populations. (A) Quantification of F480+ cells within the infected lesions of AS-605240- and vehicle-treated mice by flow cytometry. (B) Quantification of GR1+ cells within the infected lesions of AS-605240- and vehicle-treated mice by flow cytometry. Data presented as average number of F480+ (A) and GR1+ (B) cells +/- standard error of results obtained from 2 independent experiments with similar results (n= 10 mice). * indicates p< 0.05 as determined by an unpaired Students’ *t* test.
Figure 3. 6

A. Lesion

- Vehicle
- AS-605240

Total F4/80+ cells per mouse (x10^3)

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* p < 0.05

B. Lesion

- Vehicle
- AS-605240

Total GR1+ cells per mouse (x10^3)

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<td>AS-605240</td>
<td><img src="image6" alt="Graph" /></td>
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</table>

* p < 0.05
Figure 3.7. Quantification of Th1/Th2 cytokine production by draining lymph node cells of AS-605240-treated and control mice by ELISA. Eight weeks post-infection, the draining lymph nodes of *Leishmania mexicana*-infected mice were removed and the cells stimulated *ex vivo* with *Leishmania mexicana* antigen (LmAg; 20 µg/mL) for 72 hours at 37°C/5% CO₂. Supernatants were collected and analyzed for Th1-associated cytokines (A) IL-12p70 and (B) IFN-γ and Th2-associated cytokines (C) IL-4 and (D) IL-10 by ELISA. Data are expressed as mean cytokine concentration (pg/mL) +/- standard error from 3 independent experiments (n= 9-15 mice per group) with similar results. * indicates p< 0.05 as determined by an unpaired Students’ t test.
Figure 3. 7

(A) IL-12

(B) IFN-γ

(C) IL-4

(D) IL-10

- Vehicle
- AS-605240
Figure 3.8. Effect of AS-605240 treatment on regulatory T cells in *Leishmania mexicana*-infected C57BL/6 mice. Quantification of CD4+ FoxP3+ regulatory T cells within the lesions (A) and draining lymph nodes (B) of vehicle- and AS-605240-treated mice. Nine weeks post-infection, infected mice were sacrificed for flow cytometric analysis of CD4+ FoxP3+ cell populations within the lesions and draining lymph nodes. Data shown are the average numbers of CD4+ FoxP3+ regulatory T cells per mouse from each treatment group and represents collective data from 3 independent experiments with similar results. * indicates p< 0.05 as determined by an unpaired Students’ t test.
Figure 3. 8

A. Lesion

B. Lymph Nodes
Figure 3.9. Effect of AS-605240 on IL-10 production by CD4+ FoxP3+ regulatory T cells in vitro. (A) Quantification of secreted IL-10 in supernatants by sandwich ELISA. (B) Quantification of intracellular IL-10 (IL-10-APC+ cells) by intracellular cytokine flow cytometry. Naïve CD4+ FoxP3+ T cells were sorted from the lymph nodes and spleens of C57BL/6 FoxP3-EGFP reporter mice by FACS and cultured (5 x 10^5 cells) with anti-CD3/CD28 (1 µg- and 2 µg-, respectively; Biolegend®, San Diego, California, USA) stimulation for 24-48 hours in the presence or absence of 1.25 µM AS-605240. Results shown in (A) are combined results from 3 independent experiments (n= 10 mice per group) with similar results. Data shown in (B) are results from 1 of 2 independent experiments with similar results.
Figure 3.9

A. Secreted IL-10

B. Intracellular IL-10
Figure 3.10. Effect of PI3Kγ-deficiency on regulatory T cell populations in C57BL/6 mice. Quantification of CD4+ FoxP3+ regulatory T cells in the (A) spleens and (B) lymph nodes of C57BL/6 wild type and C57BL/6 PI3Kγ−/− mice. Spleens and lymph nodes were isolated from naïve C57BL/6 WT and PI3Kγ−/− mice, washed, and stained for regulatory T cell markers CD4 and FoxP3 using FITC-conjugated anti-CD4 and PE-conjugated anti-FoxP3 antibodies, respectively. Data presented are representative of results of 1 of 2 independent experiments (n = 4 mice per group) with similar results.
Figure 3.11. Evaluation of therapeutic efficacy of AS-605240-treatment to that of standard treatment with Pentostam® sodium stibogluconate (SSG). (A) Quantification of parasite burdens in mice following early treatment with saline (vehicle), AS-605240 (15mg/kg), SSG (15 mg/kg), or a combination AS-605240+SSG. Treatment began 2 weeks post-infection and continued for 14 days. Six weeks post-infection, infected mice were sacrificed, the infected ears removed, separated, and parasite burdens determined by limiting dilution analysis. (B) Quantification of parasite burdens in mice following delayed treatment with saline (vehicle), AS-605240, SSG, or a combination of AS-605240+SSG. Treatment began 6 weeks post-infection after clinical onset of disease (formation of visible, ulcerating lesions) and continued for 14 days. Ten weeks post-infection, infected mice were sacrificed, ears removed, and parasite burdens within the infected lesions determined by limiting dilution analysis. Data presented as mean log parasite dilution +/- standard error for each group and are collective of 2 independent experiments with similar results (n= 10 mice per group). * indicates p< 0.05 as determined by a Mann Whitney U prime test.
Figure 3. 11
Figure 3.12. Histopathological analysis of *Leishmania mexicana*-infected lesion from vehicle-, AS-605240-, Pentostam® sodium stibogluconate (SSG)- and combination AS-605240 + SSG-treated C57BL/6 mice. Mice were infected with 1000 stationary phase *Leishmania mexicana* promastigotes into the left ear dermis. Mice were treated 2X daily (i.p.) with (B) AS-605240 (15 mg/kg), (C) SSG (15 mg/kg), (D) or a combination AS-605240 + SSG. Control mice (A) received injections with 1X sterile saline. Mice were sacrificed 10 weeks post-infection, the infected ears removed, and tissues fixed in 10% buffered formalin. Data shown are representative hematoxylin and eosin stained histology images of *Leishmania mexicana*-infected lesions from 1 of 2 independent experiments with similar results.
Continued
Figure 3.12 Continued

C. SSG

D. AS-605240+SSG
CHAPTER 4

Future Directions

4.1 PI3Kγ AND F4/80 IN CUTANEOUS LEISHMANIASIS:

F4/80 has long been used as a murine macrophage-specific cell surface maker, however the physiological role of this seven transmembrane spanning receptor has remained largely unknown. F4/80 is expressed by a wide variety of macrophage subsets, including Kupffer cells in the liver, microglial cells in the brain, as well as cortical macrophages, red pulp macrophages, and medullary macrophages in the thymus, spleen, and lymph nodes, respectively (257). Interestingly, immature dendritic cells within the epidermis (Langerhans cells) also express the F4/80 antigen (258) however expression is reduced as cell mature and migrate to secondary lymphoid tissues.

Structural analysis of the F4/80 glycoprotein revealed an extracellular region consisting of seven repeating epidermal growth factor (EGF)-like domains followed by a ~300 base pair spacer linking the extracellular portion of the receptor to a seven transmembrane spanning region (anchoring the receptor in the membrane) containing several serine/threonine residues. For this reason, F4/80 belongs to the family of GPCR hormone receptor super-family (257).
Macrophages and dendritic cells down-regulate the expression of F4/80 upon activation. Immature Langerhans cells express F4/80 in the epidermis but decrease expression as these cells mature and travel from the skin to the lymph nodes. Similarly, F4/80 is expressed at low levels by circulating monocytes and is completely absent from marginal zone and metallophilic macrophages within the spleen (257). These findings suggest that F4/80 is likely involved in macrophage maturation and adhesion to the tissues. Investigations into other related EGF family receptors and their ligands also suggest that F4/80 may play a role in mediating cell-cell interaction (integrin binding), however the natural ligand(s) for F4/80 remain unknown and require further investigation (257, 258).

Mice deficient of F4/80 (F4/80−/− mice) are viable, fertile, and do not show any apparent defect in immune function (i.e. F4/80−/− mice remain healthy even when maintained under non-pathogen free conditions) (259). The ‘wild type’ phenotype observed in F4/80−/− mice has made the identification of an overt biological role for F4/80 difficult. A recent study by Lin et al. 2005 however, has suggested a role for F4/80 in the development of effector regulatory T cells in an experimental model of peripheral tolerance involving CD8+ cells (259).

Using an experimental model of anterior chamber associated immune deviation (ACIAD), Lee et al. 2005 identified an important link between F4/80 expression on antigen presenting cells (APCs) and the development of CD8+ regulatory T cells capable of inducing peripheral tolerance and suppressing antigen-specific delayed type hypersensitivity responses. In both ACIAD and oral tolerance models, F4/80−/− mice failed
to generate protective CD8+ regulatory T cells. Furthermore, peripheral tolerance in F4/80+/− mice could only be restored by adoptive transfer of F4/80+/+ APCs (259). Taken together, these findings strongly suggest that F4/80 likely plays an important role in regulating components of the adaptive immune response.

PI3Kγ is highly expressed by macrophages and neutrophils, and unlike its Class IA PI3K counterparts (PI3Kα, PI3Kβ, and PI3Kδ), PI3Kγ activation occurs downstream of GPCR by association with Gβγ subunits of heterotrimeric G proteins. Because F4/80 is a known member of the GPCR super-family, we investigated the effect of PI3Kγ inhibition on the expression of F4/80 by macrophages. Interestingly, inhibition of PI3Kγ by AS-605240 was found to result in the decreased expression of F4/80 by macrophages, both in vitro (Figure 4.1) and in vivo (Figure 4.2). No change in receptor expression was detected for CD68, CD11b, CD18, CD16/32 (See Chapter 2 and 3) suggesting that the decrease in F4/80 receptor expression observed in AS-605240-treated cells was specific for this particular receptor and not merely a general effect of AS-605240 on the expression of cell surface receptors by macrophages. In line with these findings are findings by Lin et al. 2005 demonstrating comparable levels of expression of the macrophage-specific receptors CR3 (complement receptor 3), class-A scavenger receptor, CD68, and sialoadhesin (CD169) between F4/80−/− and wild type mice (259).

Alternatively activated macrophages have been shown to induce suppressive T cell responses and induce CD4+ type 1 regulatory T cell development in vitro (260). The ability of macrophages to induce regulatory T cell development is dependent upon the expression of surface receptors such as F4/80 and CTLA-4 on macrophages and T cells,
respectively, as well as by secreted products such as IL-10 and indoleamine 2,3 dioxygenase (261). The induction of T cells with regulatory T cell/suppressive capacity was found to be dependent upon cell-cell contact, and removal of macrophages from \textit{in vitro} co-culture studies abolished the development of suppressive T cells \textit{in vitro}. These findings, along with those by Lin \textit{et al.} 2005, suggest that F4/80 may mediate cell-cell interactions between macrophages and resting naive T cells to promote the development of regulatory T cells and the suppression of immune responses.

CD4+CD25+ regulatory T cells have been shown to mediate susceptibility to cutaneous \textit{Leishmania major} infection in BALB/c mice, and depletion of these cells renders mice resistant to infection. If F4/80 receptor is in fact required for the development of regulatory T cells \textit{in vivo}, one may expect that F4/80\textsuperscript{-/-} mice may exhibit increased resistance against \textit{Leishmania major} infection. Although CD4+CD25+ regulatory T cells do not appear to mediate susceptibility to \textit{Leishmania mexicana} infection, F4/80 may contribute to susceptibility to \textit{Leishmania mexicana} by promoting the development of IL-10-secreting suppressive CD4+ type I regulatory T cells. In fact, the immunosuppressive CD4+ type I regulatory T cells generated by co-culture with alternatively activated macrophages were found to be unrelated to CD4+CD25+ regulatory T cells, further suggesting that F4/80 may contribute to the development of suppressive immune responses by numerous mechanisms involving the generation of distinct suppressive T cell populations.

In the future, it would be interesting to determine whether the reduced expression of F4/80 by AS-605240-treated cells/mice contributes to the protective phenotype
observed in these mice and whether PI3Kγ-deficiency promotes resistance to *Leishmania mexicana* infection via mechanisms involving F4/80 expression by APCs. These questions should be relatively easy to address in future studies using AS-605240 and PI3Kγ-deficient mice. It would be interesting to see whether C57BL/6 PI3Kγ−/− mice exhibit a defect in the development of suppressive regulatory-type T cells and whether APCs from these mice exhibit a defect in F4/80 expression. If PI3Kγ−/− mice are found to be impaired in their ability to generate regulatory/suppressive T cells, it would be interesting to see if the resistance PI3Kγ−/− mice might be abolished by adoptive transfer of F4/80+/− APCs into these mice. If so, these findings would suggest that PI3Kγ may contribute to pathogenesis of cutaneous leishmaniasis by driving the expansion of non-protective, IL-10 secreting, regulatory-type T cells via mechanisms involving F4/80 expression on APCs.

4.2 PI3-KINASES AND Fcγ RECEPTOR AND COMPLEMENT RECEPTOR (CR) 3-MEDIATED PHAGOCYTOSIS:

*Leishmania* spp. are obligate intracellular protozoan parasites of mammals, and parasite survival and replication within the host requires that parasites are rapidly taken up by host cells where they may evade destruction by components of the host immune response. *Leishmania* spp. lack the necessary machinery for active penetration and entry into host cells, and therefore are limited primarily to infecting professional phagocytes such as neutrophils, macrophages, and dendritic cells with macrophages acting as the definitive target cell for *Leishmania* parasites.
*Leishmania* spp. have been shown to interact with host cells via several cell surface receptors including Fcγ receptors, mannose receptor (262), TLRs (263), and receptors for various components of the complement cascade (98, 145) however the two primary receptors associated with facilitating *Leishmania* parasite entry into host cells and establishment of successful infection are Fcγ receptors (FcγR), and complement receptor 3 (CR3) (94, 98, 145).

CR3 (also known as Mac-1; αMβ2) is a heterodimer consisting of CD11b and CD18 and mediates phagocytosis of iC3b-opsonized particles by professional phagocytes including macrophages, neutrophils, and dendritic cells (98). Like all members of the β2 integrin family, CR3 plays an important role in mediating adhesion of leukocytes to the extracellular matrix. The significance of β2 integrins in leukocyte function is evident in human diseases (Leukocyte Adhesion Deficiency; LAD) associated with genetic and/or functional loss of CD18, the common β2 chain of all β2 integrins (264).

CR3 plays an essential role in innate immunity by mediating leukocyte adhesion, phagocyte migration, and phagocytosis (264). Signaling by CR3 is known to activate intracellular signaling kinases including MAPK and PI3K pathways, and is associated with activation of the Rho family of small guanine nucleotide-biding proteins (GTPases) which mediate signaling involved in cytoskeletal reorganization (264).

Activation of CR3 is essential for the full activation of neutrophils and monocytes and their capacity for antimicrobial effector functions including phagocytosis, cell migration, and degranulation. ‘Inside-out” signaling leading to the activation of CR3 has been suggested to occur via two distinct mechanisms. The first mechanism occurs via a
PI3K-dependent pathway involving actin and cytoskeletal reorganization induced by receptor clustering and ligation of co-receptors, such as Fcγ receptors to transduce activating signals leading to conformational ‘activating’ changes in CR3. The second mechanism of CR3 activation occurs independent of PI3K and involves signals induced by GPCR activation such as receptors for fMLP and chemokines.

Neutrophils from CD11b−/− mice exhibit impaired adhesion, phagocytosis, and respiratory burst, demonstrating the significance of CR3 in regulating numerous aspects of the host innate defense against microbial pathogens. In fact, CR3 is the most highly expressed complement receptor on macrophages and neutrophils. CR3 alone mediates binding to iC3b-opsonized particles, however CR3 may also work in cooperation with CR1 to bind C3b-bound particles and convert C3b to iC3b by way of the intrinsic cofactor I activity associated with CR1. Although CR3 is most well known as the phagocytic receptor for iC3b component of the complement cascade, CR3 has also been shown to mediate binding of microbial products such as lipopolysaccharide (LPS), Mycobacterium tuberculosis polysaccharide, Klebsiella pneumoniae acylpolygalactoside, as well as Leishmania spp. lipophosphoglycan (LPG) (264).

Fcγ receptors (FcγRs) are almost exclusively type I transmembrane glycoproteins (with the exception of human FcγRIIA) belonging to the immunoglobulin (Ig) super family and are expressed on several cell types including macrophages and neutrophils (265). FcγRs mediate binding and internalization of IgG-opsononized microbes or other particles. Mice and humans express both activating (FcγRI, FcγRIII) and inhibitory
FcγRs (FcγRIIb) and together these receptors mediate ligand binding and downstream signal transduction to regulate cellular responses (266).

FcγRs bind to the constant region of IgG molecules and initiate downstream signaling transduction events that mediate 1) the internalization of IgG-opsonized immune complexes into intracellular phagosomal vesicles, 2) the production of antimicrobial effector such as NO and ROS responsible for microbial killing, 3) increased antigen presentation by MHC complexes, and 4) the production of various cytokines and chemokines. Activation of activating FcγRs requires ligand binding (via the α chain) to induce receptor clustering. The clustering of receptor complexes within the membrane induces tyrosine phosphorylation of specific ITAM residues within the cytoplasmic tails of the common gamma chain and provides docking sites for the recruitment and activation of downstream signaling kinases that promote immune response. In contrast, activation of inhibitory FcγR leads to phosphorylation of ITIM residues within the cytoplasm ultimately leading to the recruitment and activation of protein phosphatases such as SHIP that negatively regulate activating signals within the cell.

Phagocytosis involves rearrangement of the cytoskeleton, and both CR3 and FcγR-mediated phagocytosis involves activation and signaling by the Rho family of small GTPase to induce actin polymerization and changes in the cytoskeleton. Whereas FcγR-mediated phagocytosis requires the tyrosine kinases Src and Syk, CR3-mediated phagocytosis occurs independently of signaling by these tyrosine kinases (267, 268). Furthermore, CR3-mediated uptake has typically been described as occurring via a ‘sinking’ type mechanism with little-to-no extension of the plasma membrane and
internalization into tight-fitting ‘zippered’ phagosomes (268). More recent studies, however, have suggested that uptake of iC3b-opsonized particles via CR3 involves the formation of long, thin actin-rich pseudopodia that extend around the particle to mediate internalization (267). FcγR-mediated phagocytosis on the other hand has been shown to occur via the formation and elongation of membrane-derived pseudopodia and internalization of particles into more ‘spacious’ phagosomes.

Phagocytosis of large IgG-opsonized immune complexes occurs via Fcγ receptors expressed on the surface of macrophages and neutrophils and is mediated by signaling kinases including members of the Src family of kinases as well as PI3-kinases. FcγR-mediated phagocytosis of large immune complexes, such as IgG-opsonized erythrocytes, is abrogated in cells treated with pan-PI3K inhibitors such as wortmannin, demonstrating a critical role for PI3-kinases and PtdIns(3,4,5)P3 production in mediating uptake of large, IgG-opsonized immune complexes. In contrast, the uptake of small immune complexes such as nutrients and extracellular molecules occurs via endocytic mechanisms that occur independent of PI3K activation (269).

Protein tyrosine kinases have been well established in mediating signaling involved in IgG-mediated phagocytosis and endocytosis, processes which are impaired by inhibitors of tyrosine kinases such as Genistein (269). Interestingly, whereas phagocytosis of IgG-opsonized erythrocytes is largely dependent upon tyrosine kinase activity, IgG-mediated endocytosis appears to occur independently of tyrosine kinase activity with inhibitors of tyrosine kinases having only a modest effect on endocytic uptake of IgG immune complexes (269). These findings illustrate that the intracellular
signaling events initiated during phagocytosis and endocytosis differ and warrant further investigation into the molecular mechanisms involved in internalization of distinct immune complexes mediated by different phagocytic receptors.

The classical model of FcγR activation is thought to involve cytoskeletal reorganization and the clustering of ligand-bound receptors in the plasma membrane. The clustering of FcγRs brings the associated cytoplasmic ITAM-containing γ chains in close proximity to one another and to adaptor molecule(s)/signaling complexes at the 'phagosomal cup’. Activation of activating FcγRs requires phosphorylation of tyrosine residues present within ITAMs by Src family of tyrosine kinases. Phosphorylated ITAMs provide docking sites for downstream adaptor kinases containing SH2-domains, such as Syk which are recruited to and activated downstream of activated FcγRs. Activation of Syk is associated with the activation of numerous downstream signal pathways involving Ca2+ mobilization, activation of protein kinase C (PKC), PI3-kinases, Erk, MAPK pathways, and the activation of Rho family of GTPases (265, 269).

Numerous studies have demonstrated a critical role for Syk tyrosine kinase in mediating phagocytosis of large immune complexes by macrophages and neutrophils (269-272). Phagocytosis of IgG-opsonized immune complexes is abrogated in cells treated with anti-sense Syk kinase oligodeoxynucleotides as well as in Syk−/− mice (269, 270). Similarly, overexpression of Syk in cells expressing FcγRs markedly enhances IgG-mediated phagocytosis in vitro. In contrast, inhibition of Syk has been shown to have no effect on FcγR-mediated endocytosis of small IgG immune complexes and are consistent with findings demonstrating a limited role for tyrosine kinases in FcγR-mediated
endocytosis (269). Syk has been shown to directly interact with Class I PI3K via phosphorylated tyrosine residue 317 (pTyr317) on Syk (271). In human monocytes, incubation with IgG-opsonized erythrocytes has been shown to enhance the association of Syk with PI3K (p85 regulatory subunit) whereas this association was not observed in FcγR-mediated endocytosis. Interestingly, Syk is not required for phagocytosis of latex beads or the gram-negative bacteria *Escherichia coli* by primary mouse macrophages (272). These findings illustrate that the molecular mechanisms of phagocytosis downstream of distinct phagocytic receptors are differentially regulated and utilize distinct signal transduction pathways to mediate internalization.

Phagocytosis of opsonized and non-opsonized particles mediated by either CR3 or FcγRs is dependent upon Class I PI3K activity. This is evidenced by the observation that treatment of macrophages with pan-PI3K inhibitors wortmannin and LY294002 block phagocytosis of IgG- and complement-opsonized particles, non-opsonized zymosan, and erythrocytes (215, 228, 266). Several studies have demonstrated that PI3K activity and the production of PtdIns(3,4,5)P₃ is induced upon particle binding at the ‘phagosomal cup’ and accumulates on forming phagosomes (230, 267). The specific role of individual Class I PI3K isoforms in mediating phagocytosis downstream of distinct phagocytic receptors however remains unclear.

Phagocytosis of microbial pathogens has been suggested to involve cooperation between multiple phagocytic receptors. Unlike FcγR-mediated phagocytosis which is induced solely by ligand binding and receptor clustering, CR3-mediated phagocytosis requires prior activation to induce uptake of bound particles and has been suggested to
occur via both PI3K-dependent and independent mechanisms. Activation of CR3 and phagocytosis of pathogens such as *Leishmania* spp. and *Mycobacterium tuberculosis* has been shown to involve cross-talk between CR3 and other phagocytic receptors including other complement receptors, mannose receptor, scavenger receptors, and Fcγ receptors (273). CR3 ligation has been shown to either enhance or inhibit FcγR-mediated phagocytosis by distinct FcγR isoforms. Similarly, inhibitory antibodies against CR3 significantly impair FcγR-mediated phagocytosis of IgG-opsonized particles. The cooperative interaction between CR3 and other phagocytic receptors suggests that distinct and complex intracellular signaling pathways are likely involved in mediating uptake of microbial pathogens downstream of a wide array of phagocytic receptors.

A number of studies have been published identifying a role for specific Class I PI3K isoforms in mediating phagocytosis using various experimental models. A recent study by Tamura *et al.* 2009 suggests that the p110α isoform is responsible for mediating phagocytosis of zymosan by RAW 264.7 macrophages under opsonic (IgG and iC3b) and non-opsonic conditions, as well as mediating the uptake of IgG-opsonized erythrocytes *in vitro*. In this study, p110α-‘deficiency’ (by shRNA knock-down) was found to inhibit phagocytosis of zymosan by macrophages, with p110β, p110δ and p110γ-deficiency having no effect on uptake on internalization of opsonized or non-opsonized zymosan by cells *in vitro* (225). In support of these findings, a study by Lee *et al.* 2007 demonstrated that selective inhibition of p110α (using shRNA) in PMA-differentiated THP-1 monocytes resulted in impaired phagocytosis of zymosan under non-opsonic and IgG-opsonized conditions *in vitro*. 

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In contrast to the findings of Tamura et al. 2009, PI3Kα-deficiency was not found to effect phagocytosis of serum-opsonized zymosan by THP-1 cells *in vitro*. As zymosan is known to bind multiple cell surface receptors (mannose receptor, complement receptors, TLRs) to induce phagocytosis, it remains unclear as to the specific receptor responsible for zymosan uptake in the absence of PI3Kα. Interestingly, pre-treatment of cells with mannan was found to reduce the uptake of non-opsonized zymosan to levels comparable to those observed in p110α-deficient cells but had no effect on uptake of serum-opsonized zymosan. These findings suggest that phagocytosis of serum-opsonized zymosan may occur via CR3 and that phagocytosis mediated by this receptor may involve signaling by Class I PI3-kinases other than PI3Kα (274).

To add to the complexity of our understanding of PI3-kinases and phagocytosis are studies demonstrating a role for PI3Kβ in phagocytosis by murine bone marrow derived and peritoneal macrophages. In a study by Leverrier et al. 2003, microinjection of p110-isoform selective inhibitory antibodies against PI3Kβ, but not PI3Kα or PI3Kδ, was found to significantly impair uptake of apoptotic cells as well as IgG-opsonized erythrocytes by murine macrophages, suggesting that the Class IA PI3Kβ isoform is the predominant PI3K isoform involved in mediating clearance of apoptotic cells and IgG-opsonized particles by mouse macrophages (275). The seemingly controversial results regarding the role of distinct PI3K isoforms in mediating phagocytic uptake highlight the complex nature of PI3K signaling in phagocytosis of distinct particles (bacteria, zymosan, erythrocytes, apoptotic cells, etc) mediated by specific receptors as well as illustrate the differences in signaling components/pathways between mice and men.
In this document we identify a pivotal role for the Class IB PI3Kγ isoform in mediating uptake of *Leishmania mexicana* parasites into macrophages and neutrophils. PI3Kγ, along with the Class IA PI3Kδ isoform, is highly expressed by leukocytes and is a well-known regulator of actin polymerization and cytoskeletal reorganization during macrophage and neutrophil chemotaxis. Using both genetic and pharmacological tools, we demonstrate a critical role for the Class IB PI3Kγ isoform in mediating uptake of *Leishmania mexicana* parasites into macrophages and neutrophils both *in vitro* and *in vivo*. Although our results clearly demonstrate an important role for PI3Kγ in mediating uptake of *Leishmania mexicana* parasites into macrophages and neutrophils, the specific mechanisms by which PI3Kγ mediates parasite entry into host cells and the role of distinct PI3K isoforms in mediating phagocytosis downstream of specific phagocytic receptors remains unclear.

Within the mammalian host, *Leishmania* parasites are readily opsonized by natural antibodies and complement components and are dependent upon/utilize receptor-mediated phagocytic uptake for entry into macrophages and neutrophils and parasite survival within the host. In future studies it would be interesting to assess the role of distinct PI3K isoforms in mediating phagocytosis of *Leishmania* parasites downstream of FcγRs and CR3, the two most important phagocytic receptors implicated in *Leishmania* spp. entry into phagocytes, specifically.

*Leishmania mexicana* axenic amastigotes are readily opsonized with IgG when incubated in sera obtained from *Leishmania mexicana*-infected animals (Figure 4.3). Similarly, tissue amastigotes isolated directly from the lesions of infected animals are
also opsonized by IgG (Figure 4.3). Using isoform-selective kinase inhibitors of each Class I PI3K isoform, it should be possible to identify the specific PI3K isoforms involved in mediating IgG-mediated *Leishmania mexicana* amastigote entry into macrophages via FcγR. Interestingly, promastigotes incubated in sera from infected animals are not efficiently opsonized by IgG (Figure 4.4), suggesting that FcγR-mediated phagocytosis is not the primary mechanism of promastigote entry into macrophages during the early stages of infection. Furthermore, *Leishmania mexicana* axenic amastigotes incubated in sera from naïve (uninfected C57BL/6) mice were not found to bind IgM, whereas IgM-binding was observed for promastigotes incubated with naive mouse serum (Figure 4.5).

We have confirmed that both *Leishmania mexicana* promastigotes, as well as amastigotes, can be efficiently opsonized by iC3b using human sera deficient of C5 complement component (Figure 4.6). Using isoform-selective inhibitors of each Class I PI3K isoform, we should be able to characterize the effect of isoform-selective inhibition of each Class I PI3K isoform in mediating CR3-mediated phagocytosis of iC3b-opsonized *Leishmania mexicana* promastigotes as well as axenic amastigotes into THP-1 monocytes/macrophages *in vitro*. Based on our findings and previous findings by others, we predict that PI3Kγ is a critical regulator of CR3 signaling and phagocytosis of *Leishmania* parasites into macrophages. Furthermore, it is not unlikely that CR3/PI3Kγ may cooperate with other receptors (FcγR) and Class IA PI3K isoforms (PI3Kα, PI3Kβ, PI3Kδ) to mediate phagocytosis of *Leishmania* parasites into macrophages and neutrophils. The availability of isoform-selective kinase inhibitors, p110 isoform-
specific shRNA constructs, isoform-selective anti-p110 inhibitory antibodies, and genetically modified mice should provide ample tools for in depth investigation and characterization of the role of distinct Class I PI3K isoform in mediating parasite entry into host cells.

4.3 ANALYSIS OF PI3K ISOFORMS, SIGNALING PATHWAYS/TRANSCRIPTION FACTORS INVOLVED IN CUTANEOUS LEISHMANIASIS:

We have recently identified a role for the Class IB PI3K isoform in mediating susceptibility to cutaneous *Leishmania mexicana* infection by promoting the recruitment of inflammatory cells to the site of infection and by mediating uptake of *Leishmania mexicana* into macrophages and neutrophils. Using genetically modified mice deficient of the p110γ isoform (PI3Kγ−/− mice), as well as the PI3Kγ isoform-selective kinase inhibitor AS-605240, we show that functional deficiency (either genetic or pharmacological) of PI3Kγ increases host resistance to *Leishmania mexicana* infection and that targeting PI3Kγ using isoform-selective kinase inhibitors is effective in reducing disease progression and disease-associated pathology in susceptible C57BL/6 mice. These studies clearly implicate the Class IB PI3Kγ isoform as an important component in determining disease outcome in cutaneous leishmaniasis caused by *Leishmania mexicana*.

Interestingly, a recent study by Lin et al. 2009 has shown that the Class IA PI3Kδ isoform mediates susceptibility to cutaneous infection with *Leishmania major* (201) however the mechanisms by which PI3Kδ mediates susceptibility to *Leishmania major*
differ from the mechanisms by which PI3Kγ-mediates susceptibility to *Leishmania mexicana*. Inhibition of PI3Kγ was found to significantly impair parasite entry into macrophages and neutrophils both *in vitro* and *in vivo*. Treatment of *Leishmania mexicana*-infected mice with the PI3Kγ isoform-selective inhibitor AS-605240 was found to reduce disease progression as assessed by decreased lesion development and reduced parasite burdens within the infected lesions of treated mice. The draining lymph node cells of AS-605240-treated mice were significantly impaired with regards to IL-10 production in response to *Leishmania mexicana* antigen stimulation *ex vivo*. IL-10 production by CD4+ CD25+ FoxP3+ regulatory T cells was not impaired by AS-605240, suggesting that regulatory T cells are not the source of non-protective IL-10 production to mediate susceptibility to *Leishmania mexicana* and are in consistent with earlier studies demonstrating that CD4+ CD25- FoxP3- T cells are responsible for mediating IL-10 production and susceptibility to cutaneous *Leishmania mexicana* infection in C57BL/6 mice (256).

In contrast, PI3Kδ was found to promote susceptibility to cutaneous *Leishmania major* infection by promoting the expansion, tissue homing, and effector function (IL-10 production) of suppressive CD4+ CD25+ FoxP3+ regulatory T cells (201). In line with our findings and findings by other, PI3Kδ-mutant macrophages were not impaired in their ability to internalize *Leishmania major* parasites. Taken together, these findings demonstrate that Class I PI3Ks are important for disease progression and pathogenesis of cutaneous leishmaniasis and are likely intimately involved in determining disease outcome. Furthermore, these findings suggest that distinct PI3K isoforms are involved in
mediating numerous aspects of the host immune response against *Leishmania* infection and that different species of *Leishmania* parasites may preferentially utilize distinct PI3K isoforms to promote establishment of successful infection.

The identification of specific Class I PI3K isoforms induced by *Leishmania mexicana* infection should provide insight into the contribution of distinct Class I PI3K isoforms involved in mediating the host immune response to cutaneous *Leishmania mexicana* infection. To identify which PI3K isoforms are induced in macrophages upon *Leishmania mexicana* infection, BMDMs will be infected with *Leishmania mexicana* parasites and the expression of each Class I p110 catalytic isoform determined by RT-PCR. The complete sequence of each Class I p110 catalytic subunit is available on Genbank® and will be used to design primers specific for individual PI3K p110 isoforms. Based on our previous findings, we anticipate that *Leishmania mexicana* infection of macrophages will result in increased expression of the PI3Kγ isoform. The identification of Class IA PI3K isoforms induced by *Leishmania mexicana* infection of macrophages by RT-PCR may identify additional Class IA PI3K isoforms involved in mediating parasite uptake into macrophages and/or regulating macrophage immune responses to *Leishmania mexicana* infection. RT-PCR analysis of expression of specific Class I PI3K isoforms by draining lymph node cells of *Leishmania mexicana*-infected animals may also identify specific PI3K isoforms involved in regulating the adaptive immune response in cutaneous *Leishmania mexicana* infection. Similarly, it may be interesting to determine whether the Class I PI3Ks induced upon infection with *Leishmania major* in C57BL/6 mice are similar to those induced by *Leishmania*
mexicana or whether the distinct immune responses elicited by different Leishmania species involves differential activation of distinct PI3K isoforms.

In vivo, the differentiation of human monocytes into macrophages is initiated by monocyte migration into the tissue and integrin-mediated adhesion to the extracellular matrix. The differentiation of monocytes into macrophages is associated with increased expression of specific cell surface receptors such as CD11/CD18. STAT1 has been shown to be the predominant transcription factor activated during the early stages of monocyte/macrophage differentiation and to bind to promoters of genes associated with differentiated macrophage such FcγRI, ICAM-1, and IRF-1 (276). Integrin-mediated adhesion to fibronectin and lammannin alone has been shown to induce monocyte differentiation and to induce STAT1 activation. These findings suggest that STAT1 may be involved in signaling by CD11b/CD18 integrins and activation of CR3-mediated phagocytosis.

Recent studies suggest that STAT3 may be a negative regulator of phagocytosis by macrophages. A recent study by Yuan et al. 2011 has identified an important role for Caveolin-1 (Cav-1) in mediating protective immune responses against infection with the gram-negative bacteria Pseudomonas aeruginosa. Cav-1-deficiency was associated with increased mortality, profound destruction of lung tissue, systemic dissemination of bacteria, and enhanced production of pro-inflammatory cytokines IL-6, TNFa, and IL-12 as compared to Cav-1+/+ WT mice (277). The increased susceptibility induced by Cav-1-deficiency was associated with the activation of STAT3 and the activation of NF-κB pathways leading to enhanced cytokine production and superoxide production within the
lungs, liver, and kidneys. Interestingly, macrophages obtained from Cav-1-deficient mice were found to be significantly impaired in their ability to internalize *Pseudomonas aeruginosa* as compared to wild type mice (277). A recent study by Mancuso *et al.* 2011 has reported that stimulation of alveolar macrophages with the adipose-derived hormone leptin markedly enhanced the ability of macrophages to phagocytose *Streptococcus pneumoniae* in cells expressing a mutant leptin receptor unable to activate STAT3 (278). Taken together, these findings suggest that STAT3 may negatively regulate phagocytosis by macrophages and may be involved in the attenuated phagocytic response observed in PI3Kγ deficient- and inhibitor-treated mice.

Although we have identified PI3Kγ as a critical mediator of *Leishmania mexicana* entry into macrophages, the mechanism(s)/signaling pathways involved in PI3Kγ-mediated phagocytosis is not known. *Leishmania* spp. are known to manipulate numerous host signaling pathways to promote parasite survival within the host including alteration of Jak/STAT, AP-1, CREB, and NF-κB pathways. In the future, it would be of interest to identify the transcription factors and specific signaling kinases activated in response to *Leishmania mexicana* interaction with macrophages. Comparative analysis of signaling pathways activated by *Leishmania mexicana* in the presence or absence of the PI3Kγ isoform-selective kinase inhibitor AS-605240 is likely to provide additional information regarding the mechanism(s) by which PI3Kγ mediates parasite entry into host cells. These studies should be easy to perform, as antibodies suitable for western blot analysis of phosphorylated (activated) protein kinases/transcription factors are commercially available and have been used extensively to identify the activation state of specific
signaling kinases and transcription factors. Furthermore, antibodies specific for the p110γ subunit of PI3Kγ are commercially available for use in immunoprecipitation assays and may provide a powerful tool for identifying additional signaling molecules that interact with PI3Kγ to induce *Leishmania mexicana* entry into macrophages.

4.4 MATERIALS AND METHODS:

**PARASITES:**

*Leishmania mexicana* (MNYC/BZ/62/M379) was maintained by serial passage of amastigotes inoculated subcutaneously (s.c.) into the shaven rumps of C57BL/6 mice. Amastigotes were obtained from the infected lesions and grown *in vitro* in M199 media (GIBCO® Invitrogen™ Carlsbad, California, USA) supplemented with 10% FBS and 1% Penicillin/Streptomycin (GIBCO® Invitrogen™ Carlsbad, California, USA) at 26°C without CO₂ to generate *Leishmania mexicana* promastigotes for both *in vitro* and *in vivo* studies. *Leishmania mexicana* axenic amastigotes were grown *in vitro* from promastigotes cultured in Schneider’s *Drosophila* media (GIBCO® Invitrogen™ Carlsbad, California, USA) pH=5.2 supplemented with 10% FBS, 1% Penicillin/Streptomycin at 33°C/5%CO₂ for 10-14 days.

**CELL CULTURE AND ISOLATION:**

Bone marrow-derived macrophages (BMDMs) were obtained from bone marrow-derived (BMD) cells isolated from the femurs and tibias of C57BL/6 wild type mice. BMD cells were cultured in RPMI 1649 cell culture media (GIBCO® Invitrogen™, Carlsbad,
California, USA) supplemented with 10% FBS and 1% Penicillin/Streptomycin, and 20% supernatants from in vitro-cultured L929 cells at 37°C/5% CO₂ for 7-10 days in vented 75 cm² CELLSTAR® cell culture flasks (Greiner Bio-One, Frickenhausen, Germany).

ANA-1 macrophages were a generous gift from Dr. Danuta Radzioch from McGill University, Montreal, Quebec. ANA-1 macrophage cells were grown in 75 cm² tissue culture plates in High Glucose Dulbecco’s Modified Eagle Media (DMEM) (GIBCO® Invitrogen™, Carlsbad, California, USA) supplemented with 20% FBS and 1% Penicillin/Streptomycin at 37°C/5% CO₂.

ANTIBODIES AND REAGENTS:

Leishmania mexicana parasites were fluorescently labeled with 5(6)-carboxyfluorescin diacetate N-succinimidy l ester (CFSE) (Fluka BioChemika, Buchs, Switzerland) for all uptake studies. Parasites were washed in 1X PBS (GIBCO® Invitrogen™, Carlsbad, California, USA), re-suspended at concentration = 1-10 x 10⁶/mL, and labeled with CFSE (1:1000) at 37°C for 20 minutes. PE-conjugated anti-F4/80 (Biolegend®, San Diego, California, USA) was used to quantify F4/80 expression (F4/80+ cells) on macrophages by flow cytometry. FITC-conjugated anti-IgG (Biolegend®, San Diego, California, USA) was used to quantify IgG-bound Leishmania mexicana parasites by flow cytometry. iC3b-opsonization was quantified using an murine monoclonal anti-human iC3b antibody (QUIDEL®, San Diego, California, USA) followed by a FITC-conjugated anti-mouse IgG (Biolegend®, San Diego, California, USA) secondary antibody for detection. PE-
conjugated anti-mouse IgM (Biolegend®, San Diego, California, USA) was used to determine IgM-binding by flow cytometry. iC3b-opsonization was carried out in human C5-deficient serum (Sigma Aldrich®, Saint Louis, Missouri, USA).

**IN VITRO ANALYSIS OF F4/80 EXPRESSION:**

For flow cytometric quantification of F4/80 expression by macrophages *in vitro*, cells were plated in 12 or 24 well tissue culture plates (3.5 x 10^5/mL) and infected with CFSE-labeled parasites at a ratio of 7:1 parasites: cells in the presence or absence of AS-605240 (1.25 µM) for 1-3 hours at 33°C/5% CO₂. Cells were washed to remove extracellular parasites and/or excess drug and stained with PE-conjugated anti-F4/80 antibody for quantification of F4/80 expression (F4/80+ cells) by ANA-1 and BMDMs flow cytometry.

**IN VIVO ANALYSIS OF F4/80 EXPRESSION:**

Dorsal subcutaneous pouches were created on the shaven backs of C57BL/6 wild type mice by injection with 3 mL of sterile air. These pouches were injected with thioglycollate (2 mL) to recruit macrophages into the pouches. Ninety-six hours later, these pouches were infected with 4 x 10^6 CFSE-labeled *Leishmania mexicana* parasites in the presence of either AS-605240 (15 mg/kg) or vehicle for 1-3 hours. Following infection, mice were sacrificed and cells isolated from the pouches by lavage. Cells were washed and stained with a PE-conjugated anti-F4/80 antibody for quantification of F4/80 expression (F4/80+ cells) by flow cytometry.
OPSONIZATION PROTOCOLS:

*Leishmania mexicana* parasites were washed 3X in 1X sterile PBS to remove excess media and serum by centrifugation at 3000 RPM for 5 minutes at 4°C. Parasites were counted and adjusted to 5-10 \times 10^6/mL and resuspended in 1/10 dilution of serum obtained from 6-8 week *Leishmania mexicana*-infected C57BL/6 mice or C5-deficient human serum. Parasites were incubated at room temperature for 30 minutes with shaking. Parasites were then washed 3X to remove excess serum, resuspended in 250 µl of 1X PBS, and stained with 1 µl PE-conjugated anti-mouse IgM or FITC-conjugated anti-mouse IgG for quantification of IgM and IgG binding by flow cytometry. Controls were incubated in serum from naïve (uninfected) C57BL/6 mice (NMS) or serum free M199 cell culture media. For iC3b opsonization, *Leishmania mexicana* parasites were labeled with a mouse anti-human iC3b monoclonal antibody followed by a FITC-conjugated anti-mouse IgG secondary antibody for detection.
Figure 4.1 Effect of AS-605240 on F4/80 expression by macrophages *in vitro*. Quantification of F480+ cells by flow cytometry. Flow cytometric analysis of F4/80 expression by ANA-1 macrophages (A) and C57BL/6 BMDMs (B) treated with AS-605240 (1.25 µM) or DMSO (vehicle). Macrophages were treated with AS-605240 or vehicle and infected with *Leishmania mexicana* promastigotes at 33°C/5% CO₂ for 1-3 hours. Following infection, cells were washed with PBS to remove extracellular parasites and/or excess drug and stained for the macrophage cell surface marker F4/80 for quantification of F4/80+ cells by flow cytometry. Macrophages were infected at a ratio of 7:1 parasites to cells. Data shown are representative results from 1 of 3 independent experiments with similar results.
Figure 4.1

A. **Vehicle**

- 46.1% F480+ cells
- MFI: 805

B. **AS-605240**

- 21.3% F480+ cells
- MFI: 395

**B.**

- MFI (Vehicle): 751
- MFI (AS-605240): 424

**Count**

PE-F480
Figure 4.2. Effect of AS-605240 on F4/80 expression in vivo. Flow cytometry analysis of F4/80+ cells using air pouch model of cellular trafficking. Dorsal, subcutaneous pouches were created on the shaven backs of C57BL/6 mice by injection with sterile air. The pouches were injected with 2 mL of sterile thioglycollate for 96 hours to induce macrophage infiltration into the pouches. These pouches were then injected with CFSE-labeled Leishmania mexicana amastigotes (A) and promastigotes (B) in the presence of AS-605240 (15 mg/kg) or saline vehicle for 3 hours. Following infection, the mice were sacrificed and cells isolated from the pouches by lavage. The cells were washed in PBS and stained with a PE-conjugated anti-F4/80 antibody for quantification of F4/80+ cells by flow cytometry. (A) Quantification of parasitized macrophages (CFSE+/F4/80+) in vivo by flow cytometry (note drop in F4/80 expression). (B) Histograms depicting effect of AS-605240 on F4/80 expression (% cells and MFI) by granulocytes in vivo by flow cytometry. Data shown are representative of results from 1 of 3 independent experiments (n= 9 mice per group) with similar results.
Figure 4. 2

A.

Vehicle

AS-605240

B.

Vehicle

AS-605240

37.2% F480+ cells
MFI: 251

9.88% F480+ cells
MFI: 146
Figure 4.3 Analysis of IgG-opsonization of *Leishmania mexicana* amastigotes by flow cytometry. *Leishmania mexicana* axenic amastigotes were incubated in a 1/10 dilution of serum obtained from *Leishmania mexicana*-infected C57BL/6 or naïve (uninfected) mice (NMS). Tissue amastigotes were isolated directly from the lesions *Leishmania mexicana*-infected C57BL/6 mice. Parasites were washed in PBS and labeled with a FITC-conjugated anti-mouse IgG antibody for determination of IgG-binding to parasites. Data presented is representative of results obtained from 1 of 3 independent experiments with similar results.
Figure 4.

- Naïve C57BL/6 serum (NMS)
- Tissue amastigotes
- *L. mexicana*-infected serum

FITC-anti-IgG
Figure 4.4. Determination of IgG binding to *Leishmania mexicana* promastigotes by flow cytometry. (A) Determination of IgG binding to *Leishmania mexicana* promastigotes opsonized in serum obtained from naïve (uninfected) C57BL/6 mice. (B) Determination of IgG binding to *Leishmania mexicana* promastigotes opsonized in serum obtained from *Leishmania mexicana* infected C57BL/6 mice. Following incubation, *Leishmania mexicana* parasites were stained with a FITC-conjugated anti-mouse IgG antibody for determination of IgG binding by flow cytometry. Data shown are representative of results from 1 of 4 independent experiments with similar results.
Figure 4. 4

A. 

- Serum free
- Naive C57BL/6 serum (NMS)

B. 

- Serum free
- *L. mexicana*-infected mouse serum
Figure 4.5. Determination of IgM binding to *Leishmania mexicana* parasites by flow cytometry. *Leishmania mexicana* promastigotes (A) and axenic amastigotes (B) were opsonized in serum obtained from naïve (uninfected) C57BL/6 mice. *Leishmania mexicana* parasites incubated in cell culture media devoid of serum were used as controls. Following incubation, *Leishmania mexicana* parasites were labeled with a PE-conjugated anti-mouse IgM antibody for determination of IgM binding by flow cytometry. Data shown are representative of results from 1 of 3 independent experiments with similar results.
Figure 4.6. Determination of iC3b-opsonization of *Leishmania mexicana* parasites.

*Leishmania mexicana* promastigotes (A) and axenic amastigotes (B) were incubated in C5-deficient human serum. *Leishmania mexicana* parasites incubated in cell culture media devoid of serum were used as controls. Following incubation, parasites were labeled with mouse anti-human iC3b antibody followed by a FITC-conjugated anti-mouse IgG antibody for determination of iC3b deposition by flow cytometry. Data shown are representative of results obtained from 1 of 2 independent experiments with similar results.
Figure 4.6

A.

- Serum free
- C5 deficient human serum

B.

- Serum free
- C5 deficient human serum
REFERENCES


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