Targeting Phosphoinositol 3 Kinase γ in the treatment of cutaneous leishmaniasis caused by 
*Leishmania Mexicana* 

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By 

Patrick Reville 

The Ohio State University 
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Project Advisor: Professor Abhay R. Satoskar
Introduction

Phosphoinositol-3 kinases (PI3Ks) are a family of cytoplasmic enzymes integral for cell growth, survival, differentiation and chemotaxis that can be divided into three different classes\(^1\)-\(^4\). Members of all three classes phosphorylate the D3 hydroxyl group of the inositol 6 carbon-ring contained in the membrane lipid phosphatidylinositol. This ring is polar and exposed to the cytoplasm, allowing it to serve as a substrate for the production of second messengers required for numerous signaling pathways\(^5\).

Class I PI3K can be further divided into subclasses A and B. Class IA consists of isoforms \(\alpha\), \(\beta\), and \(\delta\) and differs from Class IB, isoform \(\gamma\). Both subclasses consist of a heterodimer formed of a catalytic (p110) and a regulatory (p85, p55, or p50) subunit, which associate upon ligation of the upstream receptors specific for each ligand. Phosphorylation of a receptor tyrosine kinase’s (RTK) phospho-YXXM motif provides a docking site for association and subsequent activation of Class IA PI3Ks\(^6\),\(^7\). Until the p110 catalytic subunit associates with one of the regulatory subunits, there is only minimal kinase activity\(^8\).

PI3K\(\gamma\) is activated by members of the \(G_i\) protein-coupled receptor (\(G_i\)PCR) family. Full catalytic activity of PI3K\(\gamma\) requires the formation of a unique trimer consisting of p110\(\gamma\) and p101 with the G\(\beta\gamma\) of the G\(i\)PCR\(^9\). The p110 catalytic subunits of Class IA and IB differ in a few key amino acid residues that allow them to selectively associate with the appropriate regulatory subunits, while exhibiting minimal cross-reactivity\(^10\). The p110\(\gamma\) subunit is capable of phosphorylating phosphatidylinositol (PtdIns) when not associated with the p101 subunit; however, upon association of the two subunits, p110\(\gamma\) changes its substrate specificity to phosphatidylinositol-4, 5-bisphosphate (PtdIns (4,5) P2)\(^11\). Class IA and IB PI3Ks are found in most cells, but Class IB is more prominently expressed in cells of the hematopoietic system. The
role of PI3Kγ in the chemotaxis and directed migration of neutrophils and macrophages to inflammation sites is of key interest currently, and has thus been described as the “cell compass”

PI3Kγ has been shown to be a key mediator of chemoattractant-induced macrophage chemotaxis by C5a, CXCL12, CCL5, CCL2, and CCL22, which all signal through Gi-protein coupled receptors. Macrophages lacking PI3K-γ have been shown to exhibit up to a 90% decrease in chemotactic activity in vivo.

Macrophages and neutrophils lacking PI3Kγ show decreased directionality in response to signals through GiPCRs, due to its role in directing polymerization machinery to the site of assembly. The ability of a cell to track to the inflammation site depends on the production of an intracellular gradient of PtdIns (3,4,5) P3, or PIP3. Formation of the gradient is dependent on PI3K-γ and the antagonistic phosphatases SHIP (SH2 domain-containing inositol 5-phosphatase) and PTEN (phosphatase and tensin homologue deleted on chromosome ten), D5 and D3 phosphatases respectively. PTEN shows increased localization to the trailing edge of the cell, but is excluded from the lipid raft at the leading edge. PTEN, working in tandem with positive feedback activity by Class IA isoforms at the leading edge provides for further signal amplification. The result of this intracellular PIP3 gradient is controlled cytoskeleton rearrangement required for chemotactic activity. In addition, the activity of PI3K-γ in tandem with PI3K-δ catalytic subunits in the cell and the vascular endothelium has been shown to be a key player in forming the linkages necessary for neutrophil rolling and extravasation at the site of inflammation.

The Leishmaniases are a group of tropical diseases that differ in their clinical, histological, and immunological features/characteristics. The Leishmaniases are zoonotic diseases resulting from infection with protozoan parasites of the species Leishmania transmitted
through a sand fly vector. Entry into host cells is essential for the survival and replication of obligate intracellular parasites, such as *Leishmania mexicana*, an obligate intracellular protozoan parasite that targets macrophages and neutrophils inside the mammalian host\(^\text{23}\). Although macrophages are the principle effector cells involved in eliminating *Leishmania*, hey are also the target cell used by the parasite for survival and replication within the host. Neutrophils have been suggested to act as a “Trojan Horse” in establishing *Leishmania* infection\(^\text{24}\) in that the parasite survive within these cells, using them to gain entry into macrophages without initiating an anti-leishmanial response within the host\(^\text{25, 26}\).

This study focuses on the role of PI3K\(\gamma\) in the development of *Leishmania mexicana* infection, the causative agent of New World cutaneous Leishmaniasis. *L. mexicana* causes localized, but chronic lesions in humans, while Old World *L. major* causes localized, self-healing lesions. In most mouse strains, *L. major* and *L. mexicana* infections display lesion progression similar to that found in humans\(^\text{27}\). C57Bl/6 mice display chronic, non-healing lesions upon infection with *L. mexicana*\(^\text{28}\), making them a suitable model to study human disease progression and to identify factors that contribute to containment or proliferation of *L. mexicana*.

Studies using PI3Kinase inhibitors such as Wortmannin or LY294002 show that Type I PI3Ks are involved in mediating entry of parasites such as *T. cruzi* into host cells however the precise role of each isoform is unclear\(^\text{29, 30, 31}\). In association with the aforementioned role of PI3K\(\gamma\) in chemotaxis, PI3K\(\gamma\) has been implicated in cytoskeleton reorganization. We hypothesized that this enzyme may play a role in mediating entry of *Leishmania* into host leukocytes. Therefore, the role of PI3K\(\gamma\) was examined in respect to *Leishmania* entry into phagocytes. We sought to determine whether PI3K\(\gamma\) could be a potential therapeutic target in the treatment of cutaneous leishmaniasis caused by *L. mexicana*. 
Materials and Methods.

Mice.

Breeding pairs of PI3K-γ p110 -/- (C57BL/6), generously donated by Dr. Bao Lu (Children’s Hospital, Boston, MA), were generated by deletion of ~350 base pairs at the start of exon 2 of the p110-γ gene\(^\text{32}\). This deletion was confirmed by Southern blot analysis of DNA derived from tail clips from all breeding pairs (B. L., et. al, unpublished data). Wild-type (PI3K-γ +/-) C57BL/6 mice were purchased from Harlan (Indianapolis, IN). The experiments were performed using 8- to 10-week-old sex-matched mice in a facility at the Ohio State University according to the guidelines for animal research as required by the National Institutes of Health regulations. Under these guidelines, mice were bred and maintained in ventilated microisolator cages in a specific pathogen-free facility. Any mice showing signs of distress were immediately sacrificed. Animal care facilities at the Ohio State University are fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care-International and follow the National Research Council’s Guide for the Care and Use of Laboratory Animals.

Parasites

*L. mexicana* (MNYC/BZ/62/M379) stock cultures were maintained in 129/SVE mice by subcutaneous injection of 5x10\(^6\) promastigotes into shaven rumps. Parasites for experimental studies were then isolated under aseptic conditions from mice with non-ulcerate lesions that were crushed in a Petri dish containing 5mL supplemented M199 (Gibco, Carlsbad, CA) with 10% Fetal Bovine Serum (FBS) (*Ambogen*, Texarkana, AR), 100U of penicillin/mL and 100μg streptomycin/mL (P/S) (Cambrex, Walkersville, MD) using a 40μm cell-strainer (Beckton Dickson) and 3-mL syringe plunger (Beckton Dickson). The resulting suspension was then added to tissue culture flasks (Falcon, Franklin Lakes, NJ) containing complete M199 and grown.
until confluent. A small amount of the confluent parasite solution was then added to flasks containing fresh, supplemented M199. This procedure was continued until the parasites had been subjected to 3-6 passages, resulting in metacyclic promastigotes for use in experiments. New in vivo cultures were then begun with metacyclic promastigotes as described above.

**Bone marrow-derived macrophages (BMDM).**

Femurs and tibias from 6-8 week old PI3K-γ -/- and wild-type mice were cleaned and isolated intact. Epiphyses from both ends were removed with scissors in a tissue culture hood and the marrow was removed by inserting a needle into the cavity and rinsing with PBS, pH 7.4, until no marrow remained. This suspension was centrifuged at 1550 rpm for 10 minutes and the erythrocytes contained in the resulting pellet were resuspended and lysed with 5 mL ACK lysis buffer (150mM NH₄Cl, 10mM KHCO₃, Na₂EDTA 100μM) for 3 minutes and then diluted to 50 mL with complete RPMI (10% FBS, P/S, 0.5% 2-ME). This was centrifuged as described above, the resulting pellet was rinsed twice with complete RPMI, and was resuspended in 25 mL complete RPMI. Living cells were enumerated using Trypan blue (Gibco) exclusion (1:2 dilution) on an improved Neubauer hemacytometer. Cell concentration was adjusted to 10⁶/mL and 10-30x10⁶ cells plated in 250cm² tissue culture flasks with vented caps (Corning, Corning, NY). L929 supernatant (20%) cultured in-house was added as the source of CSF-1. Macrophages were grown for 8 days or until confluent, then rinsed twice with warm PBS, pH 7.4, and loosened with a rubber scraper. The presence of mature macrophages was confirmed by flow cytometry, as described below, using CD11b conjugated FITC and F4/80 conjugated PE as markers (BD PharMingen, San Diego, CA).
In vitro PI3Kγ inhibition on BMDMs.

BMDMs were grown as described above in complete RPMI 1640 supplemented with 20% CSF-1 for 5-6 days. BMDMs (3.5 x 10^5/ml) were plated on glass coverslips in 24-well tissue culture plates and incubated with CFSE-labeled *L. mexicana* promastigotes (parasite: BMDM ratio 7:1) in the presence of 1.25 μM AS-605240 (Merk Serono, Geneva, Switzerland) or vehicle for 0-3 hours. After infection, cells were washed, fixed, and extracellular parasites were stained using a *Leishmania* specific anti-LPG monoclonal antibody followed by a secondary PE-conjugated antibody for detection. Cells were stained with DAPI, mounted on glass slides, and intracellular parasites were enumerated by fluorescence microscopy. A minimum of 1000 cells were counted at each time point.

In vitro PI3Kγ inhibition on neutrophils (PMNs).

C57BL/6 mice were injected interperitoneally (i.p.) with thioglycollate and PMNs were isolated from the peritoneal exudate. PMNs (3.5 x 10^5/ml) were plated on collagen-coated coverslips and infected with CFSE-labeled parasites (parasite: PMN ratio 7:1) in the presence of 1.25 μM AS-605240 or vehicle for 0-1 hours. Intracellular parasites were enumerated using fluorescence microscopy as described above.

In vitro PI3Kγ inhibition on human monocyte-derived macrophages (HMDMs).

Peripheral blood mononuclear cells (PBMCs) were isolated from fresh source leukocytes with Histopaque-1077 density gradient. MDMs were generated by plating 2 x 10^7 cells in RPMI 1640 without FBS. The cells were allowed to adhere for 2 hours. Non-adherent cells were washed with PBS and the remaining cells were cultured in RPMI 1640 supplemented with 5% FBS and 1% penicillin-streptomycin for 5 days to obtain HMDMs. These cells were infected
with CFSE labeled parasites (parasite: HMDM ratio 7:1) for 0-3 hours and intracellular parasites were enumerated at different time points by fluorescence microscopy as described above.

**Flow cytometric analysis**

Macrophages, BMDMs, or PMNs were recovered, then placed on ice with 5\( \mu \)L Fc block (BD Pharmingen) per genotype and incubated for a minimum of 15 minutes. Cells were then stained with FITC, PE, or APC conjugated antibodies and incubated for 45 minutes on ice, while covered with aluminum foil. Cells were then rinsed, resuspended in PBS, centrifuged at 1200 rpm for 5 minutes a total of two times. Samples were then run on a FACSCaliber (Beckton Dickson) along with unstained controls. Due to the limited number of available cells, 5000 events were recorded per tube. Populations were then gated for lymphocytes and granulocytes as appropriate. Analysis was conducted using CellQuestPro Software (Becton Dickson).

**Analysis of in vitro PI3K\( \gamma \) inhibition on treated BMDMs and PMNs by flow cytometry.**

1-5 x 10\(^5\) mouse BMDMs or thioglycollate-elicited PMNs were co-incubated with CSFE-labeled *L. mexicana* promastigotes in 6-well plates with AS-605240 or vehicle at a ratio of 7:1 (parasite-to-cell) for two hours. Cells were recovered, washed to remove extracellular parasites and stained for macrophage or neutrophil specific surface markers (CD11b and GR1, respectively) to quantify infected macrophages (CFSE+/CD11b+) or neutrophils (CFSE+/GR1+) by flow cytometry as described above.

**Analysis of in vivo PI3K\( \gamma \) inhibition on macrophages and PMNs by flow cytometry.**

Dorsal air pouches were formed in C57BL/6 mice by subcutaneous injection with 3-cc of sterile air. These pouches were then injected with either 2 ml of thioglycollate or an LPS saline solution (1\( \mu \)g/ml) in order to recruit optimal numbers of macrophages and PMNs, respectively. For macrophage uptake studies, air pouches were inoculated with 4 x 10\(^6\) CFSE-labeled
*L. mexicana* promastigotes together with AS-605240 (15mg/kg) or saline 96 hours after thiogycollate injection. Cells were isolated from the pouches 6 hours after and stained with PE-conjugated anti-F4/80 to enumerate macrophages (CFSE+/F4/80+) by flow cytometry, as described above. For neutrophil uptake, air pouches were labeled with PE-conjugated anti-GR1 and infected PMNs were quantified (CFSE+/GR1+) by flow cytometry.

**Infection protocols.**

*L. mexicana* promastigotes derived from passage 3-6 were centrifuged at 3000 rpm for 10 minutes, supernatants were then decanted and the parasites were resuspended in supplemented, fresh M199. A 1:5 dilution of parasite suspension was then made for counting purposes, using 10% formalin as the diluent. The quantity of parasites in the initial solution was determined by counting the number of parasites present using an improved Neubauer hemacytometer. Parasites were adjusted to 1-2x10^8/mL and peanut agglutinin (PNA, Sigma) was added at 10μg/mL and incubated at room temperature for 15-30 minutes. This suspension was then centrifuged at 200-x g for 10 minutes; purified *L. mexicana* metacyclic promastigotes remained suspended in the solution, while immature or unhealthy parasites precipitated out of solution. The supernatant was then centrifuged at 3000 rpm for 10 minutes and the resulting pellet was washed twice with 50 mL fresh, complete RPMI, then centrifuged as described above each time. The PNA-purified promastigotes were then counted and adjusted to a final concentration of 10^6 per mL. Mice were anaesthetized using 0.4 mL tribromoethanol (TBE) solution (Sigma), consisting of 0.5g TBE dissolved in 1 mL tert-amyl alcohol (Sigma) and 40 mL distilled water, injected intraperitoneally. Upon induction of anesthesia, 10^3 PNA-purified metacyclic promastigotes were injected intradermally using a 30-gauge needle (Becton Dickson) and glass micro-syringe (Hamilton, Reno, NV) in the left ear pinna of each mouse. Sterile PBS, pH 7.4, (Gibco) was
then dropped into open eyes to prevent them from drying out while the mice were unconscious. Lesion size was then measured weekly using a dial-gauge micrometer (Mitutoyo, Japan) as determined by subtracting the thickness of the uninfected (control) right ear from the infected left ear.

**Inhibition of PI3Kγ on course of *L. mexicana* infection**

Wild-type C57BL/6 mice were infected with *L. mexicana* as described above. Mice were treated with AS-605240 (15mg/kg/day, i.p.) or saline beginning at week 2 post-infection and stopped at week 6 post-infection. Ear lesion size was monitored as described above.

**Relative parasite dilution assay**

Infected ears were removed from sacrificed mice at 8-week time point. The two layers of skin were then teased apart using tweezers and placed inside-down on a 70μm cell-strainer in a Petri dish containing 5 mL Schneider’s Drosophila medium (Gibco) completed with 10% FBS, P/S, and 0.5% 2-mercaptoethanol (2-ME) (Gibco) and were then crushed as described above. The resulting suspension was then centrifuged at 3000 rpm for 10 minutes, the supernatant decanted off, and the pellet resuspended in 400μL complete Schneider’s Drosophila medium. Samples were added in duplicate to 96-well tissue culture plates (TPP, Trasadingen, Switzerland) at a 1:10 initial dilution and then serially diluted across the plate. Plates were incubated at 26°C for 96 hours and then read using an inverted microscope. The wells were then examined in series until no promastigotes could be found and the last well containing promastigotes was marked, giving the limiting dilution for each sample in logarithmic form.

**Histopathology.**

At week 8 post-infection, ear lesion from both groups were excised and fixed in 10% buffered formalin for 48 hours. The tissues were processed and 5 μm sections were cut. The
sections were stained by routine hematoxylin and eosin staining, coded and analyzed for pathology.

**Isotype-specific enzyme-linked immunosorbent assay (ELISA).**

At week 8 post-infection, the draining lymph node cells from vehicle-treated controls and AS-605240-treated mice were re-stimulated *ex vivo* with *L. mexicana* antigen (20µg/ml) for 72 hours and the levels of IL-4, IL-10, IL-12, and IFN-γ generated by these cells were assessed by ELISA. Each well of a 96-well flat-bottom microtiter plate (Corning) was coated with 100µL soluble *L. mexicana* antigen (5µg/mL) in PBS (pH 9.0) and incubated overnight at 4°C. Upon removal of the soluble *L. mexicana* antigen from the plate, it was incubated for 2 hours at room temperature with milk blocking solution (5g/100 mL PBS-Tween (PBS-T)). Plates were rinsed, and then plasma samples were added in duplicate (starting dilution, 1:100) and serially diluted in PBS-T across the plate, leaving the final well as a blank. Plates were incubated at 37°C for one hour then HRP-conjugated rat anti-mouse IL-4 (1: 5,000 dilution), HRP-conjugated rat anti-mouse IL-10 (1:5,000 dilution), HRP-conjugated rat anti-mouse IL-12 (1: 5,000 dilution), or HRP-conjugated rat anti-mouse INF-γ (1: 5,000 dilution) was added in a solution of 25% FBS/75% PBS and incubated for one hour at 37°C. Peroxide substrate (Kirkegaard, Gathersville, MD) was added to the wells and stopped with 5% H₃PO₄ solution after 10 minutes. Plates were read on a microplate reader (Molecular Devices, Menlo Park, CA) at 450 nm. Data analysis was conducted using SoftMax Pro software (Molecular Devices).

**Lesion parasite loads from AS-605240-, sodium stibogluconate-, and saline- treated mice.**

Wild-type C57BL/6 mice were infected with *L. mexicana* as described above. Mice were treated with AS-605240 (15mg/kg/day, i.p.), sodium stibogluconate (250mg/kg/day) (GlaxoSmithKline, UK), or saline beginning at week 2 post-infection for a period of 14 days.
Lesion parasite burdens were determined at week 8 post infection for each mouse from each group, as described above.

**Course of *L. mexicana* infection in PI3Kγ−/− C57BL/6 mice.**

C57BL/6 mice genetically deficient of the p100γ subunit of PI3K (PI3Kγ−/−) were infected as described above. Disease progression was monitored by measuring lesion growth in the ear dermis and parasite burdens at different time points, as described above. Parasite burdens were determined at weeks 3, 6 and 9 post-infection.

**T-cell proliferation assay.**

Mice were sacrificed at time points described above; spleens and lymph nodes draining the site of infection were dissected out immediately under sterile conditions. Lymph nodes and spleens were then crushed as described above. The isolated cells were centrifuged at 1200 rpm for 5 minutes, the supernatant decanted, and the resulting spleen pellet was lysed with Boyle’s solution for 5 minutes followed by dilution with 10 mL complete RPMI. The cells were then centrifuged again as described above and rinsed twice with complete RPMI. Lymph node cells were adjusted to 3x10^6/mL and spleen cells to 5x10^6/mL then 100μL were plated out in 96-well tissue culture plates (TPP) either with (100μg/mL in complete RPMI) or without (only complete RPMI) soluble *L. mexicana* antigen made as described previously. Cells were incubated under conditions described above for 72 hours, and then supernatants from each individual organ were pooled for later analysis and stored at -80°C. Cytokine specific ELISAs for IL-4, IL-10, IL-12p70, and IFN-γ were then conducted by coating flat-bottom microtiter plates with rat anti-mouse cytokine antibodies (BD Pharmingen), diluted in binding buffer (0.1M Na₂HPO₄, pH 9.0) followed by overnight incubation at 4°C. Primary antibodies were then removed without rinsing, blocked for 2 hours using 10% FBS/PBS, followed by addition of samples in duplicate and
incubation overnight at 4°C. Samples were removed and wells rinsed 3 times using PBS/Tween (Sigma) and secondary antibody (cytokine specific, biotinylated rat anti-mouse) (BD Pharmingen) and incubated for 2 hours at room temperature. Secondary antibody was removed by washing 4 times, and avidin-conjugated alkaline phosphatase (AKP) (BD Pharmingen) was added, incubated at RT for half an hour, and then washed 5 times. P-nitro-phenol phosphate (PNPP) (1 mg/mL) was dissolved in glycine buffer (pH 10.4), and then 100μL was added per well and upon development (A=0.600-1.00), plates were read at 405nm using equipment described above.

**Statistics**

Significant results were calculated using Student’s unpaired t-test, with p<0.05 considered to be significant.
Discussion

As shown by flow cytometric data, macrophage populations were reduced at the lesion site of PI3K-γ⁻/⁻ animals, despite the lack of a significant difference in circulating white blood cell counts. With nearly a 50% reduction in the total number of macrophages at each of the three time points, these data correlate well with previous data describing impaired chemotaxis in PI3K-γ⁻/⁻ macrophages.

The effect of PI3Kγ blockade on parasite uptake by mouse macrophages and neutrophils, as well as human macrophages in vitro using a small molecule, isoform selective inhibitor of PI3Kγ, AS-605240 was examined. AS-605240 effectively competes with ATP for its binding pocket on the enzyme, rendering the PI3Kγ inactive. Bone marrow derived macrophages (BMDMs) or neutrophils from C57BL/6 mice were plated on glass coverslips and incubated with CSFE-labeled L. mexicana promastigotes in the presence of AS-605240 or vehicle for 0-3 hours. After infection, extracellular parasites were stained using anti-LPG monoclonal antibody (CedarLane Manufactured Immunology Reagents) followed by PE-conjugated secondary antibody for detection. Cells were then stained with DAPI, mounted on glass slides and intracellular parasites were enumerated by fluorescent microscopy. Both mouse BMDMs and neutrophils showed a significantly reduced uptake of parasites when treated with AS-605240 when compared with vehicle-treated controls. AS-605240 was also effective in inhibiting uptake of L. mexicana by human primary macrophages. The inhibitory effect of AS-605240 on parasite uptake was confirmed by flow cytometry for mouse BMDMs and neutrophils.

The effect of PI3Kγ blockade on parasite uptake by macrophages and neutrophils was examined in vivo. Dorsal subcutaneous air pouches were created on wild type C57BL/6 mice, as described previously. These air pouches were injected with LPS to recruit neutrophils or...
thioglycollate to recruit macrophages and then received either AS-605240 or saline along with CSFE-stained *L. mexicana* promastigotes. Cells were isolated from air pouches 6 hours post infection. The cells were washed and stained with a PE-conjugated anti-Gr1 or anti-F4/80 antibody to detect parasitized neutrophils and macrophages by flow cytometry. Mice injected with AS-605240 contained fewer parasitized neutrophils and macrophages in their dorsal pouches when compared to saline treated controls.

The results above indicate that PI3Kγ inhibition by AS-605240 reduced uptake of *L. mexicana* by macrophages and neutrophils both *in vitro* and *in vivo*. The next step was to determine whether AS-605240 could be used as an effective therapeutic in the treatment of cutaneous leishmaniasis caused by *L. mexicana*. C57BL/6 mice were infected intradermally in the ear dermis with $10^3$ metacyclic *L. mexicana* promastigotes. Beginning two weeks post infection, these mice received AS-605240 (15mg/kg) or saline vehicle interperitoneally (i.p) twice daily for four weeks. Disease progression was monitored by measuring lesion sizes weekly. Throughout the course of treatment with AS-605240, *L. mexicana*-infected mice developed either no visible lesion or significantly smaller lesion when compared with saline-treated controls. However, following cessation of treatment with AS-605240 at six weeks post-infection small lesion began to develop in these mice as well. This result suggests that AS-605240 is largely responsible for inhibiting and controlling the development of lesions and of lesion growth. Eight weeks post-infection, all mice were euthanized, ear lesions were homogenized, and parasite burdens were determined by a limiting dilution assay (as described above). AS-605240 treated C57BL/6 mice contained significantly fewer parasites in their ear lesion when compared with the saline treated controls. In addition, the ear lesions from AS-605240 treated mice contained fewer inflammatory cells and parasitized macrophages. In
contrast, the lesions of control mice showed signs of ulceration and necrosis and an increased inflammatory infiltrated consisting heavily of parasitized macrophages, neutrophils, and eosinophils.

Th2-associated cytokines IL-4 and IL-10 mediate susceptibility to *L. mexicana*\textsuperscript{23,33,38,39}. The effect of AS-605240 treatment on Th1/Th2 cytokine responses was determined. At eight week post-infection, lymph node cells from AS-605240 treated or saline treated control mice were stimulated with *L. mexicana* antigen *in vitro* and IL-12p70, INF\(\gamma\), IL-4 and IL-10 levels were determined by ELISA. *L. mexicana* antigen simulated lymph node cells from AS-605240 treated mice produced significantly less Th2 associated IL-4 and IL-10 than saline treated controls. However, levels of Th1 associated cytokines IL-12 and INF\(\gamma\) were comparable in both groups. Suppression of Th2 cytokine production in AS-605240 treated mice is most likely due to an indirect effect of AS-605240 on T cells because AS-605240 does not affect Th1 or Th2 cytokine production by naïve T cells stimulated *in vitro* with anti-CD3/ant-CD28 (data not shown). These results show that AS-605240 treatment suppresses Th2 cytokine production in *L. mexicana* infected mice, which may also contribute to the controlled parasite replication observed in these mice.

Antimonials such as sodium stibogluconate (SSG) are the standard drug of choice currently employed for the treatment of cutaneous leishmaniasis in the Old and New World. However, increasing resistance of *Leishmania spp.* to these drugs is becoming more common. Although these drugs are anti-parasitic, the precise mechanism of their action is not yet clear. The efficacy of AS-605240 treatment with SSG treatment was compared in the treatment of cutaneous leishmaniasis caused by *L. mexicana*. We found that AS-605240 (15mg/kg) was therapeutically comparable to SSG (250mg/kg) in controlling parasite growth.
To further demonstrate the role of PI3Kγ in the pathogenesis of *L. mexicana* infection, C57BL/6 mice lacking the p110γ catalytic subunit of the enzyme (PI3Kγ−/−) were infected by injecting $10^3$ *L. mexicana* promastigotes into the ear dermis and infection was monitored as described above. Lesion development progressed as expected, with an initial difference between wild type C57BL/6 mice and PI3Kγ−/− mice discernable at 5 weeks post-infection, an incubation time within normal expectations. Given the known deficiencies of PI3Kγ−/− neutrophil and macrophage migration towards the source of C5a, IL-8, as well as fMLP, and numerous chemokines produced at the site of infection, it was expected that PI3Kγ−/− mice would develop smaller lesions than their wild-type counterparts. It was our hypothesis that depriving the parasites of their obligate host cell, macrophages, would lead to a lesser extent of infection; if there are fewer macrophages to present at the infection site, then there are fewer opportunities for the parasite to exploit host macrophages for reproductive purposes, effectively hindering the ability of the parasite to multiply rapidly *in vivo*. The presence of fewer leukocytes at the infection site in PI3Kγ−/− mice explains the smaller lesion size. Reduced parasite loads at the infection site of PI3Kγ−/− mice also contributed to the smaller lesion size. Analysis of leukocyte populations in infected ears confirmed previous findings as well as our initial hypothesis, that depriving the parasite of its obligate host cell would reduce the parasite load as well as the lesion size in PI3Kγ−/− mice. Lesion sizes and parasite burdens in PI3Kγ−/− mice were comparable in AS-605240 treated wild type mice.

In conclusion, we have found that blocking PI3Kγ using an isoform selective kinase inhibitor of the enzyme, AS-605240, reduces parasite entry into macrophages and neutrophils both *in vitro* and *in vivo*. Most importantly however, we show that daily treatment with the inhibitor is as effective as standard treatment with SSG at controlling parasite growth even when
administered at a 10X lower dosage. These results provide the first “proof-of-concept” that inhibiting phagocytic uptake by blocking the intracellular lipid kinase activity of PI3Kγ could be a viable therapeutic option in the treatment of infections caused by obligate intracellular pathogens. Increased drug resistance to conventional therapeutics is a significant problem in the treatment of leishmaniasis as well as other pathogens. Using this approach would circumvent this problem by targeting host signaling pathways rather than targeting the pathogen directly.
References


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Figure 1: Inhibition of PI3Kγ activity results in reduced parasite uptake by phagocytes in vitro and in vivo.

(A) Effect of AS-605240 on L. mexicana uptake by BMDMs from C57BL/6 mice. Cells were enumerated based on protocol outlined above in, in vitro PI3Kγ inhibition on BMDMs. A minimum of 1000 cells were counted at each time point. Data is from one representative experiment of three with similar results and are expressed as the average numbers of intracellular parasites per 100 cells ± SE. *, indicates a p-value of <0.05.

(B) Effect of AS-605240 on L. mexicana uptake by PMNs from C57BL/6 mice. Cells were enumerated based on protocol, in vitro PI3Kγ inhibition on PMNs, outlined above. A minimum of 1000 cells were counted at each time point. Data is from one representative experiment of three with similar results and are expressed as the average numbers of intracellular parasites per 100 cells ± SE. *, indicates a p-value of <0.05.

(C) Effect of AS-605240 on L. mexicana uptake by HMDMs. Cells were enumerated based on protocol, in vitro PI3Kγ inhibition on HMDMs, outlined above. A minimum of 1000 cells were counted at each time point. Data is from one representative experiment of three with similar results and are expressed as the average numbers of intracellular parasites per 100 cells ± SE. *, indicates a p-value of <0.05.

(D) Analysis of in vitro uptake of L. mexicana by AS-605240 treated BMDMs and PMNs by flow cytometry. Infected cells enumerated by flow cytometry as outlined above.

(E) Analysis of in vivo uptake of L. mexicana by AS-605240 treated macrophages and PMNs by flow cytometry. Infected cells enumerated by flow cytometry as outlined above.

Figure 2: Blockade of PI3Kγ activity or PI3Kγ deficiency inhibits lesion growth and enhances host resistance against L. mexicana.

(A) Effect of PI3Kγ blockade on course of L. mexicana infection. Wild type C57BL/6 mice were infected as outlined above. Treatment of AS-605240 began at 2 weeks post infection and ceased at week 6 post infection. Lesion size is determined by measuring ear thickness compared to thickness of uninfected ear, as outlined above. Data shown are from three independent experiments (n=15 in each group) and are presented as the mean lesion size ± SE. *, indicates a p-value of <0.05. Note that AS-605240 treated mice developed small lesions when treatment was stopped at week 6 post infection.

(B) Lesion parasite loads of AS-605240 and saline treated mice at 8 weeks post infection. Lesion parasite loads were characterized from infected ears as described above. Lesion parasite loads are recorded as the highest dilution yielding viable parasite for each ear. The data presented are from three independent experiments (n=15 for each group) and are presented as the mean log parasite dilution ± SE. *, indicates a p-value of <0.05.

(C) Histopathology of infected lesions from AS-605240 treated mice.

(D) Histopathology of infected lesion from saline treated mice.

(E-H) Effect of AS-605240 treatment on production of Th1/Th2 cytokines.
Cytokine levels were assessed by ELISA as described above. Data is expressed as mean cytokine levels (pg/ml) ± SE. The data represented are from 9-15 animals per group in three independent experiments with similar results. *, indicates a p-value of <0.05.

**(I) Lesion parasite loads from AS-605240-, sodium stibogluconate-, and saline-treated mice.**
Lesion parasite loads were determined at week 8 post infection for each mouse from each group as described above. Data is expressed as mean log parasite dilution ± SE. Error bars indicate standard error (SE) of the mean for compiled lesion data.

**(J) Effect of PI3Kγ deficiency on course of L. mexicana infection.**
Lesion growth data is expressed as mean lesion size (mm) ± SE. Error bars indicate standard error (SE) of the mean for compiled lesion data. *, indicates a p-value of <0.02, ** indicates a p-value of p<0.002.

**(K) Lesion parasite loads from PI3Kγ-/- mice.**
Parasite burdens were determined at 3, 6 and 9 weeks post-infection and are expressed as mean log parasite dilution ± SE. Error bars indicate standard error (SE) of the mean for compiled lesion data. *, indicates a p-value of <0.02, ** indicates a p-value of p<0.002.

**Figure 3: Effect of AS-605240 on uptake of L. mexicana amastigotes by BMDMs in vitro.**
Cells were enumerated based on protocol outlined above in, in vitro PI3Kγ inhibition on BMDMs, excepted BMDMs were infected with CSFE-labeled L. mexicana amastigotes. A minimum of 1000 cells were counted at each time point. Data is from one representative experiment of three with similar results and are expressed as the average numbers of intracellular parasites per 100 cells ± SE. *, indicates a p-value of <0.05.

**Figure 4: Effect of AS-605240 on uptake of L. mexicana promastigotes by:**

**(A) BMDMs**
Microscopy photos from Figure 1A. Intracellular parasites fluoresce green and extracellular parasite fluoresce red/yellow. Macrophages stained with DAPI fluoresce blue.

**(B) PMNs**
Microscopy photos from Figure 1B. Intracellular parasites fluoresce green and extracellular parasite fluoresce red/yellow. Neutrophils stained with DAPI fluoresce blue.

**(C) HMDMs**
Microscopy photos from Figure 1C. Intracellular parasites fluoresce green and extracellular parasite fluoresce red/yellow. Macrophages stained with DAPI fluoresce blue.
Figure 1A.

Figure 1B.
Figure 1C.

**Human Macrophages**

![Graph showing intracellular parasites/100 cells over time (min).](image)

**Figure 1D.**

![Flow cytometry analysis showing CD11c-PE vs. CFSE-L. mexicana](image)
Figure 1E.

```
E.

AS-605240

Vehicle

1.3

5.2

8.9

55.4

CFSE- *L. mexicana*
```
Figure 2A.

Lesion Diameter

- Vehicle
- AS-605240

Weeks post infection

Figure 2B.

Parasite dilution

- Vehicle
- AS-605240

Log parasite dilution
Figure 2C.

C. AS-605240

Figure 2D.

D. Vehicle
Figure 2E and F.

Figure 2G and H.
I. Parasite Burdens

Figure 2I.

J. Lesion Diameter

Figure 2J.
Figure 2K.
Figure 3.
Figure 4 A.

Vehicle

AS-605240

Figure 4 B.

Vehicle

AS-605240
Figure 4C.