Supporting Information

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SI Materials and Methods

Animals. Eight- to 10-week-old C57BL/6 mice were purchased from Harlan. The p110γ-deficient C57BL/6 mice (backcrossed 10 times) were originally a generous gift of Bao Lu (Harvard University, Boston, MA). WT C57BL/6, p110$\gamma^{-/-}$ C57BL/6, and FoxP3-EGFP C57BL/6 knock-in mice (provided by Arlene Sharpe, Harvard University, Boston, MA) were maintained in a pathogen-free animal facility at The Ohio State University in accordance with National Institutes of Health and institutional guidelines.

Parasites and Infection Protocols. *L. mexicana* (MNYC/BZ/62/M379) was maintained by serial passage of amastigotes inoculated 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) supplemented with 10% (vol/vol) FBS and 1% penicillin (20 units/ml)/streptomycin (20 μg/ml) at 26 °C without CO_2 to generate *L. mexicana* promastigotes for both in vitro and in vivo studies. *L. mexicana* axenic amastigotes were grown in vitro from promastigotes cultured in Schneider's *Drosophila* media (pH 5.2) supplemented with 10% FBS and 1% penicillin (20 units/ml)/streptomycin (20 μg/ml) at 33 °C/5% (vol/vol) CO_2 for 10–14 d. C57BL/6 WT, p110$\gamma^{-/-}$ C57BL/6, and FoxP3-EGFP C57BL/6 knock-in mice were infected by intradermal inoculation of 1×10^3 or 1×10^4 stationary phase *L. mexicana* promastigotes into the left ear dermis. Mice were killed 5–10 wk postinfection for analysis of parasite burdens and cytokine production within the ear lesions and draining lymph nodes, respectively.

Cell Culture and Isolation. BMDMs were obtained from bone marrow-derived (BMD) cells isolated from the femurs and tibias of C57BL/6 mice. BMD cells were cultured in RPMI 1640 media supplemented with 10% FBS, 1% penicillin/streptomycin, and 20% supernatant from in vitro-cultured L929 cells at 37 °C/5% (vol/vol) CO_2 for 7–10 d in 75-cm^2 cell culture flasks. PMNs were isolated from the peritoneal exudates of C57BL/6 mice injected i.p. with LPS and cultured on collagen-coated coverslips in RPMI 1640 cell culture media supplemented with 10% FBS and 1% penicillin/streptomycin at 37 °C/5% (vol/vol) CO_2. PBMCs were isolated from fresh source leukocytes with Histopaque-1077 density gradient (SigmaAldrich). Monocyte-derived macrophages were generated by plating 2×10^7 cells in RPMI 1640 without FBS. The cells were allowed to adhere for 2 h. Nonadherent cells were washed with PBS, and the remaining cells were cultured in RPMI 1640 supplemented with 5% (vol/vol) FBS and 1% penicillin (20 units/ml)/streptomycin (20 μg/ml) for 5 d to obtain HMDMs.

Antibodies and Reagents. CFSE-labeled *L. mexicana* promastigotes or axenic amastigotes, or FITC-labeled IgG-opsonized axenic amastigotes, were used for various uptake assays. For microscopy experiments, extracellular parasites were labeled using an anti-*Leishmania* lipophosphoglycan-specific monoclonal antibody (CedarLane Manufactured Immunology Reagents), followed by an Alexa Fluor 594-conjugated secondary antibody (Molecular Probes Invitrogen) for detection. Cells were labeled by DAPI staining. Phycoerythrin (PE)-conjugated anti-GR1, PE-conjugated anti-CD11b, and PE-conjugated anti-CD68 (Biolegend) antibodies were used to quantify infected macrophages and neutrophils by flow cytometry. Flow cytometric analysis of surface expression of macrophage phagocytic receptors CD11b, CD18, and CD16/32 was performed using PE-conjugated anti-CD11b, FITC-conjugated anti-CD18, and allophycocyanin-conjugated anti-CD16/32 antibodies.

Histopathology. Infected ears from infected mice were removed, separated, and fixed in decalcifying solution F (Stephens Lab) or 10% (vol/vol) buffered formalin for 7 d. The tissues were processed and embedded in paraffin, and 4- to 8-μm sections were cut. The sections were hydrated and stained by routine H&E staining.

T-Cell Proliferation Assay and Cytokine Analysis. The draining lymph nodes were removed from *L. mexicana*-infected mice 5–8 wk after infection. For T-cell proliferation assays, 3×10^6 lymph node cells were added to the wells of a 96-well, flat-bottomed tissue culture plate. Cells were stimulated with 20 μg/mL freeze-thawed LmAg or supplemented medium as a negative control. After incubation at 37 °C and 5% (vol/vol) CO_2 for 72 h, supernatants were collected from parallel cultures for ELISA quantification of cytokine production. Cultures were analyzed for production of IL-4, IL-10, IL-12, and IFN-γ (reagents purchased from Biolegend; detection limit: 3 pg/mL for IL-4 and 20 pg/mL for all others).

Statistical Analysis. The Student's unpaired *t* test was used to determine the statistical significance of values obtained. Differences in parasite titers were determined using the Mann–Whitney *U* prime test.

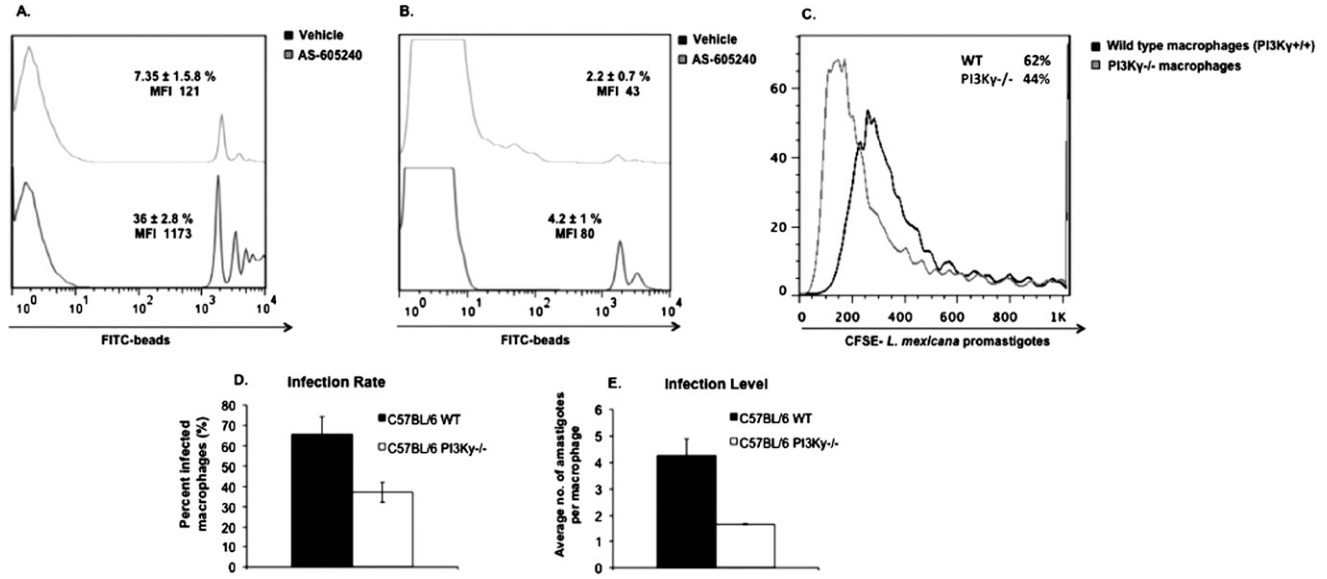

Fig. S1. PI3Kγ mediates phagocytosis by macrophages in vitro. (*A* and *B*) AS-605240 inhibits the nonspecific phagocytosis of collagen-coated fluorobeads by macrophages. BMDMs from WT C57BL/6 mice (*A*) and ANA-1 macrophages generated from WT C57BL/6 mice (*B*) were treated with 1.25 μM AS-605240 or sterile DMSO (vehicle) and incubated with latex fluorobeads (7:1 beads/macrophages) for 1 h at 37 °C/5% (vol/vol) CO_2 and analyzed by flow cytometry for quantification of bead-harboring ($FITC^+$) macrophages. MFI, mean fluorescence intensity. (*C*) WT C57BL/6 (ANA-1) macrophages and similarly generated PI3Kγ$^{-/-}$ macrophages were infected with CFSE-labeled *L. mexicana* promastigotes (7:1 parasites/macrophages) for 1 h at 33 °C/5% (vol/vol) CO_2 and analyzed by flow cytometry for quantification of parasitized ($CFSE^+$) macrophages. (*D* and *E*) BMDMs (0.5×10^6) from WT C57BL/6 mice and PI3Kγ$^{-/-}$ mice were infected overnight with 2.5×10^6 (5:1 ratio) *L. mexicana* promastigotes from stationary phase in 24-well tissue culture plates (Corning, Inc.). Infected macrophages were stained by GIEMSA (Sigma), and the infection rate and infection level were assessed by counting both infected and uninfected (100–200 total) cells and calculating the percentage of BMDMs harboring amastigotes after 72 h of stimulation. Infection levels were found by counting the average number of amastigotes per infected BMDM. Data presented are representative of results from one of three independent experiments with similar results.

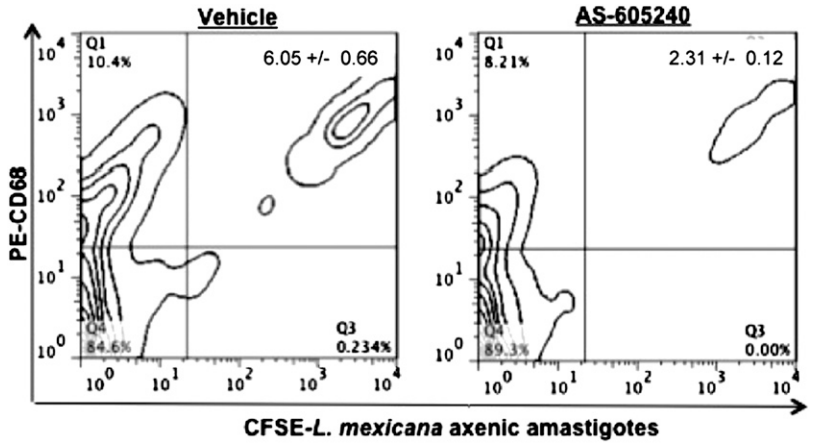

Fig. S2. AS-605240 suppresses uptake of axenically grown amastigotes into macrophages in vivo.

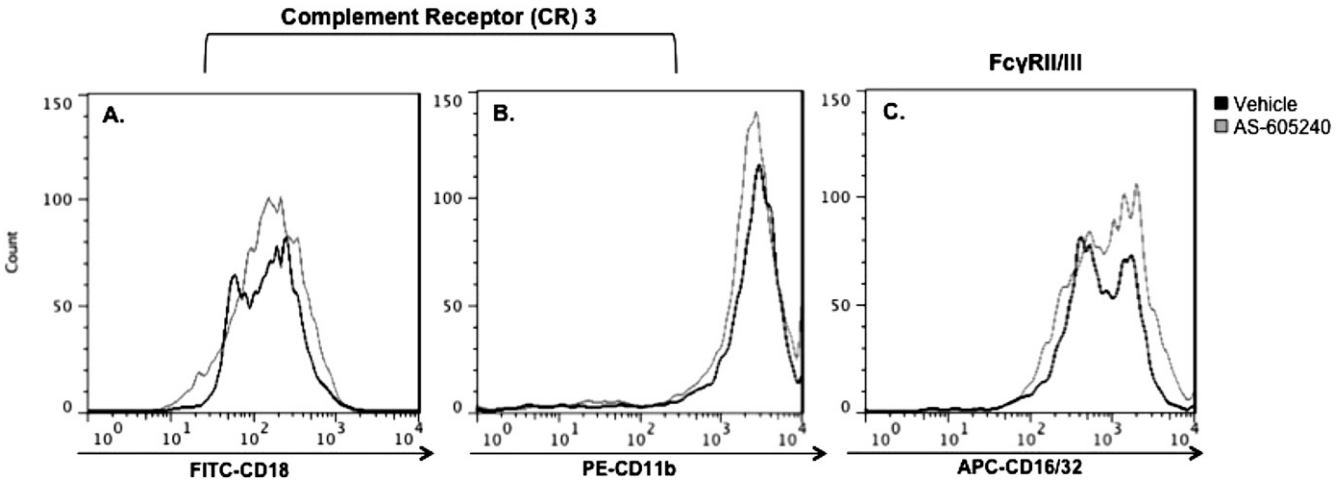

Fig. S3. Effect of PI3Kγ blockade on levels of phagocyte receptors that are involved in mediating entry of *Leishmania*. C57BL/6 BMDMs were grown in 12-well tissue culture plates and treated with 1.25 μM AS-605240 or DMSO (vehicle) for 3 h at 37 °C/5% (vol/vol) CO_2. Following incubation, cells were washed in PBS and stained for flow cytometry analysis of expression of cell surface receptors CD18 (*A*), CD11b (*B*), and CD16/32 (*C*) by macrophages. Data are representative of results from one of three independent experiments with similar results.

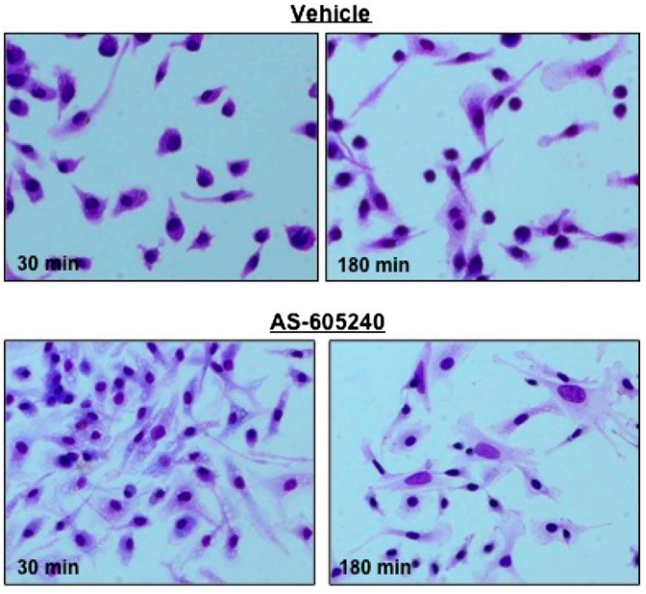

Fig. S4. AS-605240 is not toxic to macrophages. C57BL/6 BMDMs were grown on glass coverslips in 24-well tissue culture plates and treated with 1.25 μM AS-605240 or DMSO (vehicle) for various time points at 37 °C/5% (vol/vol) CO_2. Following incubation, cells were washed in PBS to remove excess drug, fixed in methanol, stained with Giemsa, and mounted on glass slides for visualization by standard light microscopy (Magnification: 100×).

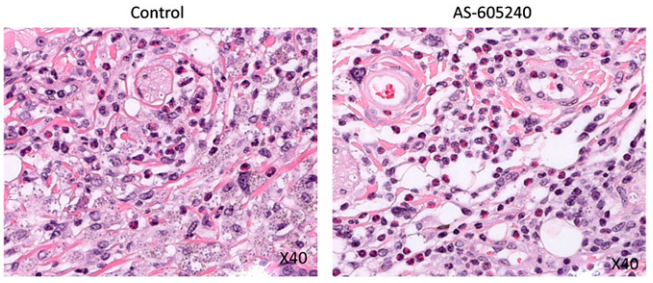

Fig. S5. Histopathology of ear lesions of *L. mexicana*-infected mice treated with (saline) controls or AS605240. The ear lesions from AS-605240–treated mice contained fewer inflammatory cells and parasitized macrophages compared with the lesions of control mice, which showed ulceration and necrosis, as well as an increased inflammatory infiltrate composed of heavily parasitized macrophages, neutrophils, and eosinophils.

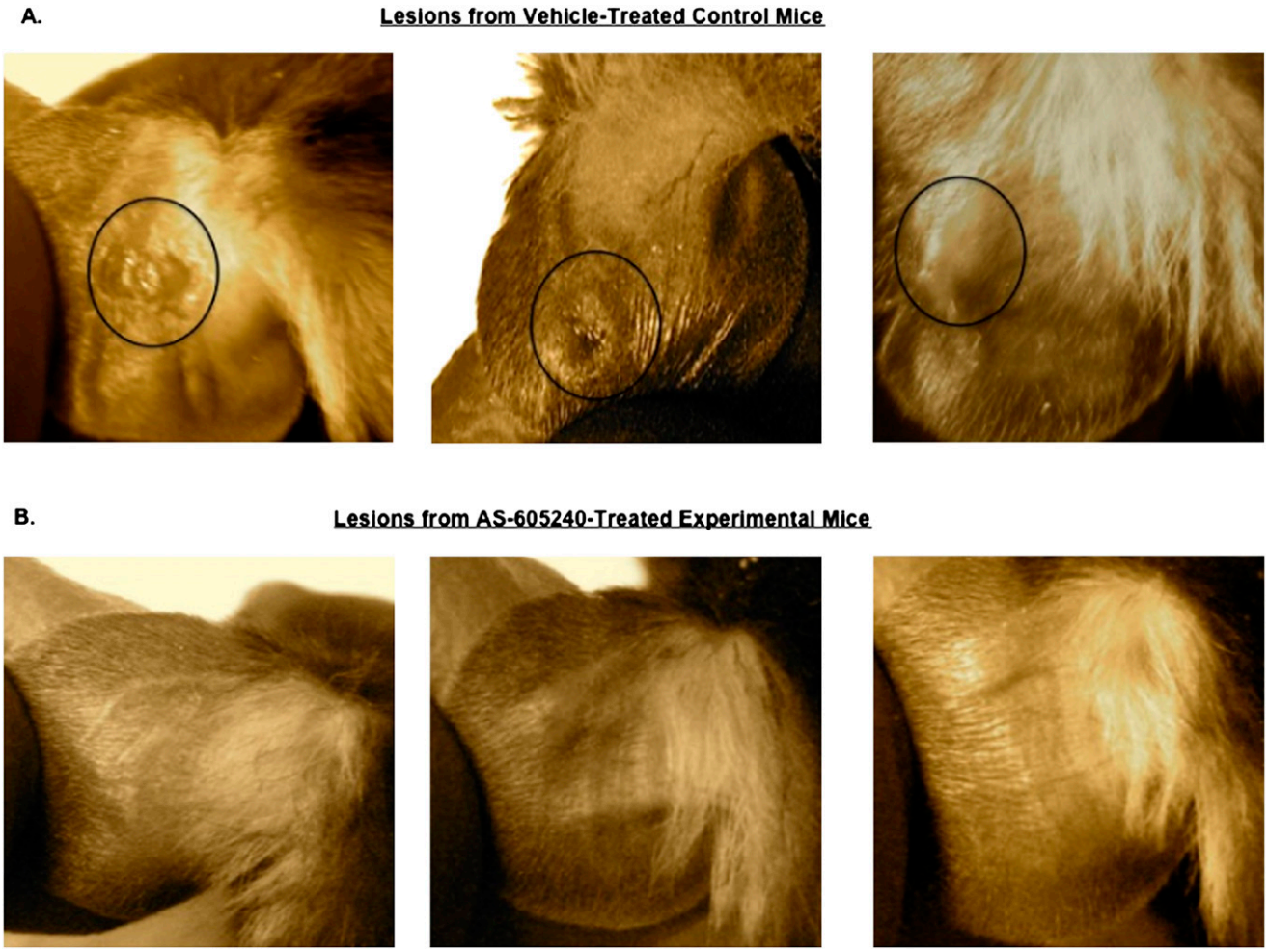

Fig. S6. Ears from *L. mexicana*-infected C57BL/6 mice treated with saline (controls) or AS-605240. Control mice develop ulcerating lesions (*A*), which are absent in mice treated with AS-605240 (*B*). Ears for three individual mice are shown.

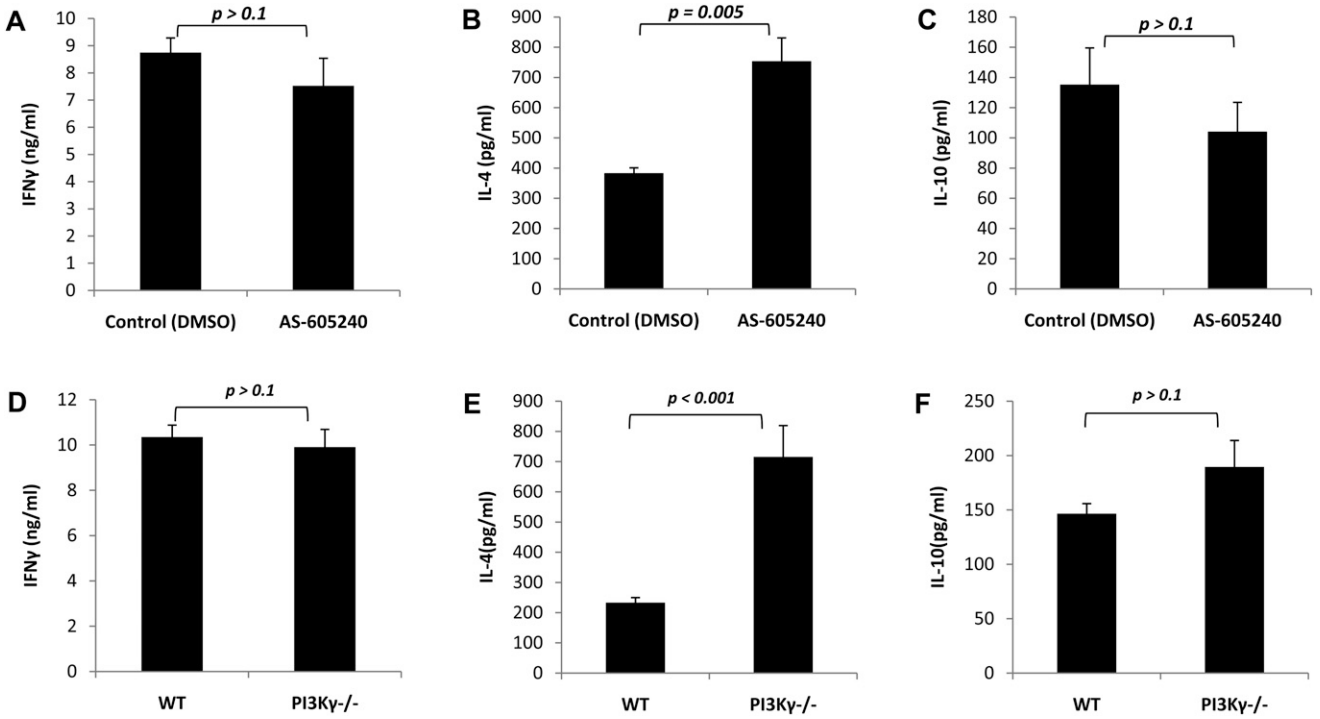

Fig. S7. Effect of PI3Kγ blockade or PI3Kγ deficiency on IFN-γ, IL-4, and IL-10 production by T cells. T cells were purified from the spleens of WT and PI3Kγ$^{-/-}$ C57BL/6 mice as described previously (1). A total of 2×10^6 WT T cells per well were plated in quadruplicate in 24-well tissue culture plates and stimulated in vitro with plate-bound anti-CD3/anti-CD28 in the presence of 1.25 μM AS-605240 or DMSO (control) for 48 h, and levels of IFN-γ (*A*), IL-4 (*B*), and IL-10 (*C*) in the supernatants were measured by ELISA. T cells from WT and PI3Kγ$^{-/-}$ C57BL/6 mice were stimulated as described above for 48 h, and levels of IFN-γ (*D*), IL-4 (*E*), and IL-10 (*F*) in supernatants were measured by ELISA. Data are from two independent experiments with identical results.

1. Barbi J, et al. (2008) PI3Kgamma (PI3Kgamma) is essential for efficient introduction of CXCR3 on activated T cells. *Blood* 112:3048–3051.