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Mannose-Capped Lipoarabinomannan from Mycobacterium tuberculosis Preferentially Inhibits Sphingosine-1-Phosphate–Induced Migration of Th1 Cells

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Chemokine receptor cross-desensitization provides an important mechanism to regulate immune cell recruitment at sites of inflammation. We previously reported that the mycobacterial cell wall glycolipid mannose-capped lipoarabinomannan (ManLAM) could induce human peripheral blood T cell chemotaxis. Therefore, we examined the ability of ManLAM to desensitize T cells to other chemotaxants as a potential mechanism for impaired T cell homing and delayed lung recruitment during mycobacterial infection. We found that ManLAM pretreatment inhibited in vitro migration of naive human or mouse T cells to the lymph node egress signal sphingosine-1-phosphate (SIP). Intratracheal administration of ManLAM in mice resulted in significant increases in T cells, primarily CCR5+ (Th1) cells, in lung-draining lymph nodes. To investigate the selective CCR5 effect, mouse T cells were differentiated into Th1 or Th2 populations in vitro, and their ability to migrate to SIP with or without ManLAM pretreatment was analyzed. ManLAM pretreatment of Th1 populations inhibited SIP-induced migration but had no effect on Th2 cell SIP-directed migration, suggesting a differential effect by SIP on the two subsets. The PI3K/akt inhibitor Ly294002 inhibited SIP-directed migration by Th1 cells, whereas the ERK inhibitor U0126 inhibited Th2 cell SIP-directed migration. These observations demonstrate that SIP-induced migratory responses in Th1 and Th2 lymphocytes occurs via different signaling pathways and suggests further that the production of ManLAM during Mycobacterium tuberculosis infection may function to sequester Th1 cells in lung-draining lymph nodes, thereby delaying their recruitment to the lung. The Journal of Immunology, 2012, 189: 5886–5895.

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Abbreviations used in this article: i.e., intratracheal(ly); LAM, lipoarabinomannan; ManLAM, mannose-capped lipoarabinomannan; PIM6, hexamannosylated phosphatidylinositol; SIP, sphingosine-1-phosphate; SIP1, sphingosine-1-phosphate receptor 1.

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Once secreted, ManLAM can interact with host cell surface receptors such as C-type lectins or the mannose receptor (13–15). Alternatively, ManLAM can incorporate directly into lipid rafts on PBMCs (16). ManLAM’s interactions with host cell receptors and membranes results in altered cellular signaling and responses. This is thought to be achieved through a steric inhibition mechanism or through direct binding of host proteins to the acyl tails of ManLAM itself, which resemble mammalian phosphatidylinositol-3,4,5-trisphosphate (17–19). A recent study demonstrated ManLAM’s ability to inhibit CD4+ T cell activation via inhibition of p56lck phosphorylation and signaling from the TCR (20). Furthermore, ManLAM stimulation prevents phagolysosomal fusion in M. tuberculosis–infected macrophages via a PI3K-dependent pathway (21, 22). Mechanistically, S1P-directed migration in endothelial cells and T lymphocytes has previously been shown to rely upon PI3K/AKT signaling pathways (23–25).

In this study, we investigated the ability of ManLAM to desensitize human and mouse T lymphocytes to CCL21 and S1P-rely upon PI3K/AKT signaling pathways (21, 22). Mechanistically, S1P-directed migration in endothelial cells and T lymphocytes has previously been shown to rely upon PI3K/AKT signaling pathways (23–25).

Materials and Methods

Ags and Abs

ManLAM, anti-ManLAM Ab (CS-35, 1:250 titer), whole irradiated H37Rv, and hexamannosylphosphatidylinositol (PIM6) were obtained through the Colorado State University.

Mice

Eight- to 12-wk-old female C57BL/6j for ManLAM experiments (Jax 000664) were housed at the Laboratory Animal Science Center at Boston University School of Medicine or the Animal Medicine facility at University of Massachusetts Medical School. Mice were administered food and water ad libitum. All experiments were performed in compliance with the U.S. Department of Health and Human Services Guide for the Care and Use of Laboratory Animals and were approved by either the Boston University Institutional Animal Care and Use Committee or the University of Massachusetts Medical Center Institutional Animal Care and Use Committee and Institutional Biosafety Committee, respectively.

Isolation of human peripheral blood T cells

All human cell studies have been approved by the Boston University Institutional Review Board and the National Institutes of Health and were conducted in accordance with the guidelines of the World Medical Association’s Declaration of Helsinki. Primary human T cells from healthy donors were isolated as described previously (26). Briefly, peripheral blood was obtained from healthy donors and the T cells isolated from theuffy coat following Hypaque Ficoll (Amersham Biosciences) density gradient centrifugation and nylon wool adherence (Polysciences). The T cells, 95% CD3+, were cultured in complete M199 (Life Technologies) until use.

Isolation of murine T cells

Mouse T cells were isolated from mixed spleen and lymph node preparations. Cells were dissociated, and RBC lysis was performed using RBC Lysing Buffer (Sigma-Aldrich) prior to selection with either nylon wool or an Invitrogen Dynal CD4–negative bead isolation kit per the manufacturer’s protocol. Mouse T cells were incubated in RPMI 1640 medium supplemented with 1% BSA, HEPES, and penicillin/streptomycin prior to use in assays (Life Technologies).

In vitro M. tuberculosis infections. Bone marrow–derived macrophages were generated through culturing bone marrows isolated from C57BL/6j mice in complete RPMI 1640 medium containing 20% L929 culture supernatant for 1 wk. A total of 4 × 105 cells plated in 24-well plates were either left in culture (control) or infected with M. tuberculosis Erdman (Trudeau Institute Mycobacterial Culture Collection) at a multiplicity of infection of 5. Cell culture supernatants were collected 48 h postinfection and were sterile filtered and frozen prior to use. Anti-ManLAM Ab (CS-35) was used to preclear either control or M. tuberculosis–infected supernatants at 4˚C overnight prior to use in chemotaxis assays. After pre-clearing, the supernatants were used to pretreat murine T cells prior to use in Transwell assays.

Migration assays

Boyden chamber assays were conducted as described previously (27). Human T cells were incubated with components from the H37Rv strain of M. tuberculosis prior to migration induced by 10 ng/ml recombinant S1P (Avanti Polar Lipids) and CCL21 (R&D Systems). Migration was conducted for 1 h using an 8 µm pore nitrocellulose filter (Neuroprobe) before the filters were fixed and H&E stained. Migration through the filter was compared with baseline migration established with media control. Quantification was accomplished by counting 5 high-power fields, and on average, 10–15 cells/field were counted for media controls. Migration was expressed as fold over media control.

Transwell assays were conducted using mouse T cells from spleens and lymph nodes. Cells were incubated in RPMI 1640 medium with 1% BSA, HEPES, and penicillin/streptomycin and then suspended at 1 × 106 cells/100 µl. The cells were incubated in Transwell chambers with 5-µm polycarbonate filters for 3.5 h prior to counting cells in the bottom chamber. Directed migration was established as more cells as compared with media control wells, which usually contained 10,000–20,000 cells. Flow cytometric analysis of CCR4 and CCR5 expression was performed on pooled cells that migrated to the bottom chambers. Migration was expressed as fold over media control.

Flow cytometry analysis

Cells were Fc blocked with anti-CD16/32 Abs (eBioscience, San Diego, CA) for 10 min prior to staining with selected Abs for 30 min on ice. The following anti-mouse Abs and isotype controls were purchased from eBioscience: CD3-FITC (clone 145-2C11), CD3-PerCP-Cy5.5 (clone 145-2C11), CD4-FITC (clone GK1.5), CD8-PE (clone 53-6.7), CCR5-PE (clone 2B11), CD49d-PE (clone 4A6), CD127-PE (clone 45-397), and CCR7-PE-Cy7 (clone 7A4), and CCR7-PE-Cy7 (clone 4B12). Anti-mouse CCR4 and CCR5 expression was accomplished using chemotaxis assays. Migration was expressed as fold over media control.

In vivo imaging experiments

Isolated T lymphocytes were labeled with QDot 565 nanocrystals per the manufacturer’s protocol (Invitrogen). Labeling was confirmed by flow cytometry, and 5 × 106 labeled T cells were injected via the tail vein into recipient 8- to 12-wk-old female C57BL/6j mice. Mice were allowed to rest for 1 h before PBS or 25 µg ManLAM was administered i.t. Mice were imaged 24 h postinstillation with the Caliper Life Sciences In Vivo Imaging System Spectrum and Living Image software version 3.2 (Hopkinton, MA) with the following settings: excitation = 430 nm, emission = 560 nm, F stop = 1, and exposure = 30 s; image depicts counts following background subtraction.

In vivo i.t. instillation of tuberculosis Ags

Mice were i.t. instilled with the tuberculosis Ags in a volume of 100 µl. Twenty-four hours following instillation, the inguinal, cervical, mediastinal, and axillary lymph nodes were isolated, and cell counts were performed. T cell numbers were determined via flow cytometric analysis of CD3 expression. Cells isolated from lymph nodes were also assessed for CCR4, CCR5, and CCR7 expression.

Th1/Th2 skewing

Mouse T cells were skewed using 3 µg/ml plate bound anti-CD3 (clone 17A2; eBioscience) and 3 µg/ml soluble anti-CD28 (clone 37.51; eBioscience) and cultured for 3 d in RPMI 1640 medium supplemented with...
10% FCS (Atlanta Biologicals), 1X nonessential amino acids, HEPES, sodium pyruvate, 2-ME, and penicillin/streptomycin (Life Technologies). Th1 cells were cultured with 10 ng/ml IL-12 and 10 μg/ml anti-IL-4 (clone 30340, R&D Systems), and Th2 cells were cultured with 10 ng/ml IL-4 and 10 μg/ml anti-IFN-γ (clone H22; R&D Systems). Cells were split on day 3, rested in plain medium 24 h prior to harvest, and harvested on days 7–8. Skewing was confirmed by IL-4, IL-5, and IFN-γ ELISA (R&D Systems).

Western blotting

Human or mouse T cells were incubated with ManLAM or vehicle control for 2 h. Cells were then washed and stimulated with S1P for 5 min prior to lysis with buffer (Cell Signaling Technology) containing protease inhibitors (Roche) and phosphatase inhibitors (Sigma-Aldrich). Samples were run on a 4–12% Bis-Tris gel (Invitrogen) and transferred to polyvinylidene difluoride membranes (Millipore). Membranes were blocked with 4% milk in TBS with 0.1% Tween 20 and probed with the following Abs from Cell Signaling Technology per the manufacturer’s protocol: phospho-AKT Ser473, total AKT, phospho-p44/42 MAPK, total p44/42 MAPK, and goat anti-rabbit HRP-conjugated secondary Ab. Detection was performed with an ECL reagent kit (GE Healthcare), and films were scanned for densitometry analysis with ImageJ Software (National Institutes of Health).

Statistical analyses

All data are presented as the mean ± SEM. Statistical analyses were performed as either Student’s t test or two-way ANOVA with Bonferroni post tests (Graph-Pad Prism software, version 5.0 for Windows).

Results

Human T cells exhibit decreased migratory responses to S1P following ManLAM treatment

To initially determine whether ManLAM could affect human T lymphocyte trafficking to or from lymph tissue, we examined the ability of human T lymphocytes to migrate to a lymph node–specific chemokine, CCL21, or to the lymph node egress factor S1P following ManLAM treatment. T cells were isolated from PBMCs from healthy human donors, pretreated with whole irradiated H37Rv M. tuberculosis, ManLAM, another M. tuberculosis cell wall component PIM6 (16), or vehicle for 2 h, washed, and placed in Boyden chemotaxis chambers. Boyden assays were performed with human cells because of reproducibility and migratory capacity of the cells, based on prior studies and experience in our laboratory (27). S1P-induced human T cell migration, but not CCL21-induced migration, was significantly inhibited following irradiated H37Rv or ManLAM treatment (\(p < 0.02\), \(p = 0.007\), optimal doses shown; Fig. 1A, 1B, see also Supplemental Fig. 1). PIM6 stimulation, used as a glycolipid control, had no effect on human T cell chemotaxis following S1P stimulation (Fig. 1C). A dose titration curve of ManLAM demonstrated that a concentration of 100 ng/ml was the lowest effective concentration of ManLAM that inhibited S1P directed migration (Supplemental Fig. 1).

ManLAM pretreatment inhibits S1P-induced AKT phosphorylation in human T cells

To determine the mechanism for inhibition of S1P-induced migration, surface expression levels of CCR7 and S1P1 were assessed via flow cytometry following ManLAM pretreatment. It was determined that there was no downmodulation of receptors from the cell surface (Fig. 2A, 2B). Because ManLAM had no effect on chemokine receptor expression, we examined signaling pathway activation in the T cells to determine the mechanism of desensitization. Western blotting was performed on cell pellets that had been pretreated with vehicle or ManLAM and then stimulated with S1P for a total of 5 min (Fig. 2C). Quantification demonstrated a 22% increase in AKT phosphorylation over baseline following S1P stimulation, which was partially blocked by ManLAM pretreatment (16% increase over baseline, representative blot from two separate experiments). Next, we wanted to determine whether this phosphorylation event was biologically relevant for S1P-induced migration. A recent study demonstrated that S1P/S1P1 signaling in T cells affected regulatory T cell development and suppressive functions through a PI3K/AKT-dependent migration pathway (23). Another study demonstrated that AKT-mediated phosphorylation of S1P1 is required for endothelial cell migration toward S1P (24). Treatment with the PI3K/AKT inhibitor Ly294002 blocked naïve human T cell migration to S1P (\(p = 0.05\); Fig. 2D).

ManLAM treatment induces decreased mouse T cell migratory responses to S1P

To determine whether the effects of ManLAM could be investigated in a murine model, we next examined the ability of ManLAM to affect in vitro migration of mouse T cells stimulated by either CCL21 or S1P. T lymphocytes were isolated from the spleen and lymph nodes of 8- to 12-wk-old female C57BL/6J mice and were pretreated with whole irradiated H37Rv M. tuberculosis for 2 h prior to use in Transwell chemotaxis assays (28). As with human cells, mouse naïve T cells that had been treated with irradiated H37Rv or ManLAM demonstrated no significant difference in their responsiveness to CCL21 but did exhibit a significant decrease in their response to S1P as compared with vehicle treated cells (\(p = 0.03\) and 0.02, respectively; Fig. 3A, 3B). The same concentration of PIM6, another M. tuberculosis glycolipid, had no effect on S1P- or CCL21-induced migratory responses (Fig. 3C).

![FIGURE 1.](http://www.jimmunol.org) ManLAM pretreatment desensitizes naïve human T cell migration to S1P. T cells from healthy donors were treated with 1 mg/ml whole irradiated H37Rv (A), 100 ng/ml ManLAM (B), or 100 ng/ml PIM6 (C) for 2 h before use in Boyden chamber assays for media control, S1P (10 nM), or CCL21 (50 ng/ml). Migrating cells were fixed and stained, and the number of T cells counted at ×400 magnification from at least 10 fields was normalized to media control. irH37Rv inhibited S1P-directed migration but not CCL21-directed migration (\(p = 0.02\); Student’s t test). (B) Pretreatment with 100 ng/ml ManLAM also blocks S1P-directed migration (\(p = 0.007\); Student’s t test). Data in (A) and (B) were obtained from three donors assayed in duplicate (mean ± SEM). (C) A dose of 100 ng/ml PIM6 had no effect on human T lymphocyte migration induced by S1P (\(n = 2\) donors assayed in duplicate, mean ± SEM).
To further test the specificity of the effect of ManLAM on S1P-induced migration, we examined the ability of an anti-ManLAM Ab to potentially neutralize the ManLAM effect. For these studies, T cell migration to S1P was assessed following pretreatment with culture supernatants from M. tuberculosis Erdman–infected mouse bone marrow–derived macrophages. Bone marrow–derived macrophages were cultured (control) or infected with a multiplicity of infection of 5 M. tuberculosis Erdman (MTB sup) for 48 h prior to harvest of supernatants. Supernatants were precleared with CS-35 anti-ManLAM Ab overnight at 4˚C, before pretreating mouse T cells for 1.5 h at 37˚C. T cells were then washed and subjected to a Transwell assay. CS-35 alone did not affect mouse T cell migration to S1P (*p = 0.05; Fig. 3D). As expected, M. tuberculosis supernatant blocked S1P-directed migration; however, supernatant that had been precleared to remove ManLAM had no effect (*p = 0.05; Fig. 3D).

To determine whether AKT was also involved in mouse T cell migratory responses to S1P, Western blots were performed as in Fig. 2C. As with human T cells, ManLAM pretreatment inhibited AKT phosphorylation in mouse T cells (representative blot from two separate experiments; Fig. 3E). Ly294002 treatment blocked mouse T cell S1P-directed migration; however, supernatant that had been precleared to remove ManLAM had no effect (*p = 0.05; Fig. 3D).

To more accurately quantify cell numbers and to assess the rate of accumulation, T cell kinetics studies were performed to determine onset and extent of the effect of a single bolus of ManLAM on T cell homing to secondary lymph tissue. Inguinal, axillary, cervical, and mediastinal lymph nodes were harvested from mice 12, 24, and 48 h following instillation of ManLAM or vehicle. Total T cell numbers were determined via CD3 staining, and maximal accumulation was observed at 24 h postinstillation (only the 24-h time point is shown; Fig. 4C). There was a 3-fold increase in T lymphocytes in the cervical lymph nodes and a 2-fold increase in T cells in mediastinal lymph nodes of mice that received ManLAM to alter in vivo T cell homing patterns in mice. T cells from lymph nodes and spleens of donor mice were labeled with Invitrogen 565 QDot nanocrystals. Five million labeled cells were injected via the tail vein into 8- to 12-wk-old recipient female C57BL/6J mice and were tracked using whole-body imaging. After 1 h, the mice were i.t. administered ManLAM or vehicle. A dose of 25 mg ManLAM was chosen to correspond to doses used in mouse Ag challenge models and to correspond with levels of ManLAM detected in sputum during live M. tuberculosis infection (29, 30).

Homing patterns and lymph node accumulation of labeled cells 24 h postinstillation were determined with the In Vivo Imaging System Spectrum. On the basis of these heat map images, it was determined that ManLAM instilled i.t. induced accumulation of T cells in areas of the chest, which likely corresponded with the mediastinal and cervical lymph nodes (Fig. 4A). To more accurately localize the recruited cells, mice were sacrificed, and the lymph nodes were excised. The cervical and mediastinal lymph nodes were visibly enlarged, whereas the inguinal and axillary nodes had no detectable difference (Fig. 4B).

**FIGURE 2.** Naive human T cell migration to S1P requires intact AKT signaling. (A) Cells pretreated with ManLAM do not modulate surface CCR7 or S1P1 expression as determined by flow cytometry (gray filled histogram, isotype control; black, PBS treated; gray, ManLAM treated; representative image). (B) Quantification of surface receptor expression as in (A) (n = 3 donors assayed in duplicate, mean ± SEM). (C) Western blotting analysis of human T cells pretreated with ManLAM or PBS for 2 h and then stimulated with S1P or vehicle for 5 min. Percent-phosphorylated AKT was determined by creating a ratio of densitometry of phospho-AKT Ser473 to total AKT (representative blot; n = 2 separate experiments with three donors). (D) Pretreatment with 10 μM Ly294002 for 1 h inhibits human T cell migration to S1P (*p = 0.05; Student t test, n = 2 donors assayed in duplicate, mean ± SEM).
ManLAM as compared with mice that received vehicle 24 h following treatment (*p = 0.04 cervical and 0.03 mediastinal, respectively; Fig. 4C). In contrast, there was no significant difference in the number of cells present in either the inguinal or axillary lymph nodes (Fig. 4C), suggesting that i.t. administration of ManLAM altered homing patterns of cells only within lymph nodes that directly drain the lungs. Cell numbers from animals treated with PIM6 were not significantly different from vehicle-treated animals (Fig. 4D). In addition, mice were instilled with LPS. LPS is a likely contaminant, and it has been shown to have effects on recruitment and/or activation of immune cells in the lungs (31, 32). As shown in Fig. 4E, instillation of LPS induced a slight decrease in the number of T cells in lung-draining lymph nodes, although this was not statistically significant.

The increase in T cell numbers in the lymph nodes could be a result of increased proliferation. To ascertain whether the increase in T cells in lung-draining lymph nodes following ManLAM instillation was as a result of T cell proliferation, cell samples from ManLAM and vehicle-treated mice were stained with propidium iodide and were subjected to cell cycle analysis. As shown in Fig. 4F, there was no difference in proliferative profiles of cells isolated from vehicle or ManLAM-treated animals, indicating that ManLAM was not inducing proliferation of these cell populations. These data agree with other published studies demonstrating that ManLAM does not have a proliferative effect and in many cases inhibits T cell proliferation (33).

LAMs from other mycobacterial species

We also tested LAMs from other mycobacterial strains to determine whether the effects we observed were unique to M. tuberculosis, Mycobacterium smegmatis LAM was assayed and found to inhibit T cell migration to both S1P and CCL21 (Supplemental Fig. 2A). Similar to ManLAM, LAM from Mycobacterium leprae inhibited T cell migration to S1P to the same extent as M. tuberculosis ManLAM (Supplemental Fig. 2A) as well as having no effect on CCL21-induced migration.

We also tested LAM from M. leprae and M. smegmatis in the in vivo i.t. model. LAM from M. smegmatis was unable to induce an effect on the number of T cells in the lymph nodes (Supplemental Fig. 2C), whereas LAM from M. leprae did induce a significant increase in cervical lymph node T cells, but the effect was not as large as that seen with M. tuberculosis ManLAM (*p = 0.02; Student t test).

**FIGURE 3.** Migratory responses of mouse T lymphocytes after exposure to tuberculosis Ags. Mouse T cells were preincubated with 1 mg/ml whole irradiated H37Rv (A), 100 ng/ml ManLAM (B), or 100 ng/ml PIM6 (C) for 2 h. Cells were washed and placed in Transwell chambers and stimulated for migration induced by media control, S1P (10 nM), or CCL21 (50 ng/ml). The assay was terminated after 3.5 h, and migration was expressed as fold change over baseline (media control expressed as 1). Data in (A)–(C) are from three independent experiments performed in triplicate (mean ± SEM; *p = 0.03, *p = 0.02; Student t test). (D) Culture supernatants from control or M. tuberculosis-infected mouse bone marrow–derived macrophages were harvested 48 h postinfection. Supernatants were precleared with CS35 anti-ManLAM Abs at 4°C overnight. Primary mouse T cells were pretreated with the supernatants for 1.5 h prior to Transwell assay. The data depicted shows migration over baseline to S1P at 10 nM. (Duplicate wells from two separate experiments; mean ± SEM; *p = 0.04; Student t test). (E) Western blotting analysis of mouse T cells pretreated with ManLAM or PBS for 2 h and then stimulated with S1P or vehicle for 5 min. Percent-phosphorylated AKT was determined by creating a ratio of densitometry of phospho-AKT Ser473 to total AKT. (Representative blot, n = 2 separate experiments). (F) Mouse T lymphocytes were pretreated with 50 μM of the PI3K/AKT inhibitor Ly294002 for 1 h prior to Transwell migration. S1P migration was significantly inhibited, whereas migration to CCL21 was not significantly affected (n = 3 experiments performed in triplicate; *p = 0.02; Student t test).
ManLAM preferentially inhibits CCR5^+ T cell migration to S1P in vitro and in vivo

To better understand the inhibitory effect of ManLAM, we assessed the phenotype of lymph node–sequestered cells following in vivo ManLAM treatment. Cells were dissociated from lymph tissue of mice that had been treated i.t. with PBS or ManLAM for 24 h and were assessed for expression of CCR4 (predominantly expressed by Th2 cells) and CCR5 (predominantly expressed by Th1 cells) by flow cytometry. The ratios of CCR5^+ to CCR4^+ (Th1:Th2) cells isolated from cervical and mediastinal lymph nodes of mice that had received ManLAM were higher than that of mice that received vehicle, with statistically significant increases in total CCR5^+ cells in vivo that were recruited to the lymph nodes following i.t. administration. Therefore, the equal numbers of T cells may result from the balance of both a net reduction of newly recruited cells and egressing cells, thereby resulting in the little increase in detectable lymphadenopathy.

ManLAM preferentially inhibits CCR5^+ T cell migration to S1P in vitro and in vivo

To determine whether changes in the percentage of CCR5^+ cells could be attributed exclusively to altered migration patterns or whether ManLAM stimulation could induce alteration in surface expression of CCR4 or CCR5, T cells were incubated in vitro with ManLAM for 2, 5.5, and 24 h prior to surface receptor analysis (these time points correspond with pretreatment, migration assays, and in vivo ManLAM administration, respectively). For all concentrations and time points investigated, there was no significant change in expression of CCR4 or CCR5 (representative data shown; Fig. 5C). These findings indicate that the significant reduction in CCR5^+ cell numbers responding to S1P was due to direct effects on migration and not on the alteration of CCR5 expression.

We also examined total cell numbers (Supplemental Fig. 2B, 2C) and ratios of CCR5^+ to CCR4^+ (Th1 to Th2) cells (Supplemental Fig. 2D, 2E) from mice that had been administered LAM from M. smegmatis or M. leprae and found no significant increases. This indicates that the preferential sequestration of CCR5^+ (Th1) cells in lymph nodes is a selective effect for M. tuberculosis ManLAM.

**Th1 and Th2 cells require different signaling pathways to migrate toward S1P**

The in vivo migration studies indicated that ManLAM was having a greater effect on CCR5^+ than CCR4^+ cell S1P migration. To more directly investigate this, we analyzed S1P-directed migration in Th1 versus Th2 cell populations. Naïve cells were skewed in vitro under Th1 or Th2 polarizing conditions, and subset purity was confirmed using Th1/Th2 cytokine analysis by ELISA. Skewed cell populations were pretreated with ManLAM or vehicle, washed, and placed in Transwell chambers. ManLAM pretreatment was able to inhibit Th1 cell migration to S1P but not Th2 cell migration (**p = 0.009; Fig. 6A). These data are consistent with reports showing that ManLAM stimulation can enhance Th2 cytokine production but diminish Th1 cytokine production (34).

ManLAM has been shown to preferentially desensitize the AKT pathway (18, 19), thereby providing a potential mechanism for the selective inhibitory effect of ManLAM on Th1 cell migration. To examine differences in AKT pathway activation, Western blotting was performed on cell pellets of Th1 and Th2 cells that had been pretreated with PBS or ManLAM, followed by stimulation with S1P (representative blots shown with average percentage of
phosphorylated protein from two separate experiments; Fig. 6B, 6C). Strikingly, Th1 cells exhibited basal phospho-AKT, whereas Th2 cells exhibited basal phospho-ERK. ManLAM pretreatment alone induced an increase in phospho-AKT over baseline but prevented cells from achieving the same extent of AKT phosphorylation as control cells stimulated with S1P (Fig. 6B). Conversely, ManLAM pretreatment resulted in enhanced ERK phosphorylation as compared with S1P stimulation alone in Th2 cells,
indicating that the cells were still responsive to the stimulus following ManLAM pretreatment (Fig. 6C).

We next investigated the functionality of the AKT and ERK pathways, which have both been shown to be important for T cell chemotaxis, in Th1 and Th2 cell migration induced by S1P. As shown in Fig. 6D, 6E, the AKT inhibitor Ly294002 significantly inhibited Th1 migration to S1P, whereas the ERK inhibitor U0126 significantly inhibited Th2 migration to S1P, with neither inhibitor significantly affecting the migratory response of the other T cell subset (*p = 0.02 and **p = 0.005). Taken together, these data indicate that S1P-induced migration of Th1 cells requires activation of the AKT pathway, whereas S1P-induced migration of Th2 cells is primarily dependent on the ERK pathway. Furthermore, *M. tuberculosis* ManLAM preferentially affects the AKT pathway, thereby inhibiting S1P-induced migration of Th1 cells.

**Discussion**

In this study, we demonstrate that a *M. tuberculosis* virulence factor, ManLAM, is capable of inhibiting T cell migration to the lymph tissue egress signal S1P. This was accomplished by interference with PI3K/AKT pathway activation. Th1 populations were preferentially affected because of their dependence on AKT activation for S1P-induced migration. It is possible that the effect of ManLAM on T cells could delay and potentially reduce initiation of Th1 effector responses in lung tissue, where even transient delays in initiation of adaptive immunity could contribute to long-term impairment for control of infection (8, 35, 36). Furthermore, these findings demonstrate a potential mechanism by which *M. tuberculosis* could foster a favorable infection environment and suggest that therapeutics that target ManLAM, such as benzothiazinones, may significantly alter Th1 cell skewing and recruitment to the lung, thereby reducing the bacterial burden (37).

We have identified that naive and Th1 cell S1P-induced migration requires the AKT pathway and that ManLAM pretreatment is able to inhibit S1P-directed migration of these populations. The selective effect of ManLAM on Th1 cells seems to rely on their propensity to use the AKT pathway as opposed to the ERK pathway: we found that Th1 cells have basal levels of phospho-AKT that are absent in Th2 populations, and conversely, Th2 cells have basal phospho-ERK that is absent in Th1 populations. These data corroborate previous studies that have shown distinct signaling events in human Th1 and Th2 cells (38) and that AKT and ERK contribute to Th1 and Th2 differentiation, respectively (39, 40). Additional studies are required to examine in more detail the mechanisms by which ManLAM desensitizes the AKT pathway.

A number of chemotaxtractants have been reported to induce desensitization of cells to subsequent migratory stimuli. Examples of this would be cross-desensitization of CCR5 by IL-16 and MIP-1β (41), CD4-induced desensitization of CXCR3 and CCR5 following IL-16 stimulation (42), and desensitization of CXCR2 and C5aR by fMLP stimulation (43, 44). ManLAM has previously been shown to induce T cell migration (45), and we now demonstrate that pretreatment with ManLAM renders T cells refractory to S1P stimulation. Most of the chemokine receptor cross-desensitization studies involved receptor-mediated desensitization, and ManLAM has been shown to interact with some cell surface receptors including C-type lectins and the mannose receptor (13–15). Future studies could determine whether ManLAM interacts with a receptor on the surface of T cells that could mediate this effect. Another potential mechanism is the direct incorporation of ManLAM into the membranes of PBMCs: Ilangumaran et al. (16) showed that ManLAM preferentially incorporates into lipid rafts where AKT activation following S1P stimulation normally occurs.

Our data suggest that ManLAM can selectively inhibit the AKT pathway without abrogating ERK signaling. ERK signaling has been shown to be caveolae independent and therefore potentially less affected by membrane integration by ManLAM (46). This difference may explain why ManLAM preferentially inhibits the PI3K pathway; however, additional studies are required to determine the mechanism by which ManLAM binds to T cells and initiates the inhibitory effect on S1P receptor signaling.

Our studies did not address effects of ManLAM on other S1P receptors expressed by other immune cells. Of note, there are five different S1P receptors that are differentially expressed on immune cells (47). Our studies focused on T lymphocytes (48), which express S1P1 and S1P3 as do B lymphocytes; however, mast cells, macrophages, and dendritic cells express S1P2 and ManLAM stimulation either through membrane integration or receptor interaction could affect cellular responses. Further examination of this phenotype could reveal preferential use of different S1P receptors on different leukocytes (49). It has also been shown that MTB inhibits sphingosine kinase activity, the enzyme responsible for production of S1P (50, 51). It is possible that reduced production of S1P in vivo, a consequence of MTB infection, could contribute to the phenotype we observe.

Previous studies have shown that 100 ng/ml ManLAM represents the lower end of a dose response curve used to inhibit CD4+ T cell activation and cytokine secretion (20, 34). In addition, human *M. tuberculosis* patient sputum has been shown to contain 1 ng/ml to 1 µg/ml ManLAM (30), although the precise concentration in lymph nodes is unknown. It has been reported that ManLAM incorporates into lipid rafts on the surface of PBMCs as rapidly as 30 min, with maximal incorporation occurring within several hours (16). Concentrations and time points used in this study correspond well with these studies, supporting the concept that ManLAM can alter the immune response in the nanogram range and within a short period of time. During the course of a live infection, it is likely that ManLAM is constantly secreted by infected macrophages, thus potentiating the sequestration of cells for an extended period of time. The spread of infection to the lymph nodes and subsequent ManLAM secretion within the confines of the lymph tissue could further contribute to inhibition of Th1 cell egress with reduced immune responses in the lung. Interestingly, *M. tuberculosis*-infected CCR5−/− mice demonstrate increased lymphocytic infiltrates in their lungs and increased proinflammatory cytokine production (52), which would be consistent with ManLAM’s ability to efficiently and selectively inhibit CCR5+Th1 cell subsets but not CCR4+Th2 subsets.

There is evidence that indicates a shift in Th1/Th2 cytokines during *M. tuberculosis* infection. Infected mice initially produce both IFN-γ and IL-4 in the lungs, but IFN-γ levels decrease, whereas IL-4 levels increase over the course of infection (53). This finding is consistent with our data indicating that ManLAM production would alter normal Th1 cell homing patterns. ManLAM has also been shown to promote secretion of Th2-related cytokines while simultaneously decreasing release of Th1 cytokines from T lymphocytes (34). As IFN-γ production by Th1 cells is considered to be host-protective, our data would support the hypothesis that shedding of ManLAM during *M. tuberculosis* infection promotes an environment that is more favorable for infection and suggests further that antibiotics targeting ManLAM, such as benzothiazinones, may significantly alter Th1 cell skewing in the lung thereby reducing the bacterial burden (37). It is likely that live MTB produce other factors that are immunoregulatory. Our data indicate that shedding of ManLAM represents a major component for induction of lymphadenopathy with selectivity for CCR5+ T cells; however, additional studies are required to more
fully understand how bacterial products can modify the immune response and promote the infection process.

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**Disclosures**

The authors have no financial conflicts of interest.

**References**


