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Inhibitory Role of Sphingosine 1-Phosphate Receptor 2 in Macrophage Recruitment during Inflammation

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Abstract

Macrophage recruitment to sites of inflammation is an essential step in host defense. However, the mechanisms preventing excessive accumulation of macrophages remain relatively unknown. The lysophospholipid sphingosine 1-phosphate (S1P) promotes T and B cell egress from lymphoid organs by acting on S1P receptor 1 (S1P₁R). More recently, S1P₅R was shown to regulate NK cell mobilization during inflammation, raising the possibility that S1P regulates the trafficking of other leukocyte lineages. In this study, we show that S1P₂R inhibits macrophage migration in vitro and that S1P₂R-deficient mice have enhanced macrophage recruitment during thioglycollate peritonitis. We identify the signaling mechanisms used by S1P₂R in macrophages, involving the second messenger cAMP and inhibition of Akt phosphorylation. In addition, we show that the phosphoinositide phosphatase and tensin homolog deleted on chromosome 10, which has been suggested to mediate S1P₂R effects in other cell types, does not mediate S1P₂R inhibition in macrophages. Our results suggest that S1P serves as a negative regulator of macrophage recruitment by inhibiting migration in these cells and identify an additional facet to the regulation of leukocyte trafficking by S1P.

Sphingosine 1-phosphate (S1P) is a bioactive lipid with roles in diverse physiologic processes including angiogenesis, lymphocyte recirculation, and vascular permeability (1–3). S1P exerts its biological effects by signaling through five G protein-coupled receptors (S1P receptors 1–5 [S1P_{1–5}R]). At the cellular level, S1PRs regulate the migration of a variety of cell types, including endothelial cells, smooth muscle cells, lymphocytes, and cancer cells. S1P₁R and S1P₂R have opposing effects on cell migration. S1P₁R is a G_i-coupled receptor that stimulates migration in endothelial cells and lymphocytes (4,5). In contrast, S1P₂R is a G_{12/13}-coupled receptor known to inhibit migration in a variety of cell types, including cancer cells, smooth muscle cells, and fibroblasts (6–10). Importantly, S1P also inhibits the *trans*-endothelial migration of neutrophils (6). The S1P₂R-mediated

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inhibition of migration involves altered Rho GTPase activity and the inositol phosphatase and tensin homolog (PTEN) deleted on chromosome 10 (8–13).

S1P is concentrated in blood (and to a lesser extent in lymph), whereas S1P levels in interstitial fluids are much lower (14). Thus, a gradient of S1P normally exists between the vascular space and interstitial fluid, forming the vascular S1P gradient. The existence of this gradient is particularly relevant to leukocytes, which rely on chemotactic gradients for homing to lymphoid organs and sites of inflammation. Indeed, the vascular S1P gradient plays a central role in lymphocyte egress from lymphoid organs, where S1P₁R has been shown to direct the chemotaxis of lymphocytes out of lymphoid tissue and toward S1P in blood and lymph (4). S1P has also been shown to be chemotactic for dendritic cells (DCs) (15–17). Consistent with a role for S1P₁R, the immunosuppressant FTY720, a functional antagonist of S1P₁R, inhibits the migration of DCs toward S1P in vitro and to lymph nodes (LNs) in vivo (15,18,19). More recently, osteoclasts and NK cells have been shown to use S1P for trafficking (20,21).

Macrophages are monocyte DCs of the innate immune system important in combating infection, wound repair, and adaptive immunity (22). Macrophages modulate immune responses, performing proinflammatory and anti-inflammatory roles by secreting cytokines and lipid mediators. They are proficient in the phagocytosis of pathogens and cellular debris, enabling them to clear infections and restore tissue homeostasis. Inherent in their role as immune regulators is the ability of the macrophage lineage to respond to immune challenge by migrating to sites of infection and inflammation. Together with varying cellular phenotypes, macrophages display a wide array of migratory behaviors. For instance, subtypes of macrophages are long-term residents in tissues, whereas others are highly motile cells generated from monocytes during inflammation. Once recruited to sites of inflammation, these motile macrophages actively drain to LNs (23). Macrophage chemotaxis is stimulated by microbial products, complement, and chemokines, allowing them to seek out pathogenic, inflammatory, and non-inflammatory foci. The chemoattractants responsible for recruiting macrophage during inflammation include the CC-motif family of chemokines. Yet, the negative regulation of macrophage migration, and the negative regulation of leukocyte chemotaxis in general, remain poorly understood.

Although S1P is known to regulate the migration of lymphocytes and DCs, the role of S1P in macrophage migration remains unknown. In this study, we characterize the role of S1PRs on macrophage migration. Using the thioglycollate (TG) peritonitis model of acute inflammation and *S1p2r* knockout mice, we show that S1P₂R negatively regulates the accumulation of macrophage during inflammation. In vitro, we show that S1P inhibits macrophage migration through S1P₂R ligation. S1P₂R signaling stimulates cAMP production and inhibits chemoattractant-induced Akt phosphorylation. However, chemoattractant-induced receptor signaling remains intact, including calcium flux and MAPK phosphorylation. In addition, protein kinase A (PKA)-selective cAMP analogs mimic the effects of S1P₂R signaling, inhibiting migration, and Akt phosphorylation. Our results demonstrate that S1P₂R can influence macrophage recruitment during inflammation, and we identify a novel mechanism for S1P₂R in regulating leukocyte migration in vivo.

Materials and Methods

Mice

S1p2r^{-/-} mice were obtained from Dr. Richard Proia (National Institutes of Health, Bethesda, MD), *Pten*^{lox/loxP} mice were obtained from The Jackson Laboratory (Bar Harbor, ME), and *Rosa26 CreERT2* mice were obtained from Dr. Guo-Hua Fong (University of Connecticut Health Center, Farmington, CT) (24). All mice were fertile and had developed

normally. Mice were genotyped as described (25,26) and housed under specific pathogen-free conditions. Experimental procedures were approved by the University of Connecticut Animal Care and Use Committee.

Reagents and Abs

The following Abs were purchased from eBioscience (San Diego, CA): APC anti-CD11b, PE anti-F4/80, PE-Cy7 anti-CD11c, and Pacific Blue anti-CD45. HBSS and DMEM were from Invitrogen (San Diego, CA), 8-Br-cAMP and fura 2 were from Calbiochem (San Diego, CA), and 8-pCPT-2'-O-Me-cAMP and 6-Phe-cAMP were from Biolog (Bremen, Germany). Recombinant mouse C5a and CXCL12 were from R&D Systems (Minneapolis, MN). Tamoxifen and fatty acid-free BSA were from Sigma-Aldrich (St. Louis, MO). TG was from Fisher (Pittsburgh, PA). S1P was from Biomol (Plymouth Meeting, PA).

TG peritonitis

Twelve-week-old mice were injected i.p. with 1 ml sterile TG (3% w/v). At indicated times after challenge, mice were euthanized by using CO₂, peritoneum lavaged with 5 ml cold HBSS, and draining *para*-thymic and nondraining ileal LNs collected. LN cell suspensions were obtained by brief dissection, collagenase digestion, and filtering through 40- μ m nylon mesh. Total leukocyte counts were determined using a hemocytometer and cell suspensions were analyzed by flow cytometry. CD45⁺ macrophage, polymorphonuclear neutrophils, and lymphocytes were delineated using forward and side scatter characteristics, CD11b, F4/80, and CD11c.

Generation of bone marrow macrophage

Bone marrow was collected by aseptically removing femurs and tibias of *S1p2r^{+/+}* and *S1p2r^{-/-}* mice and flushing with cold HBSS using a 25G needle. Cell suspensions were then centrifuged, lysed of red cells using ammonium chloride, and cultured in sterile bacterial dishes for 7 d in DMEM supplemented with 10% FBS and 20% L cell conditioned media, as a source of M-CSF. The resulting cultures were analyzed by flow cytometry for CD11b and F4/80 expression. To generate PTEN-deficient bone marrow-derived macrophages (BMDM), *Pten^{loxP/loxP}*, *Rosa26 CreER^{T2}*, and *Pten^{loxP/loxP}* mice were fed 200 mg/kg tamoxifen in corn oil by oral gavage for 5 consecutive days. One day later BMDM were cultured from total bone marrow. PTEN- and S1P₂R-deficiency had no effect on the efficiency of BMDM generation, which typically yielded ~95% CD11b⁺ F4/80⁺ cells.

Peritonitis in S1P₁R^{-/-} fetal liver chimeras

Livers from CD45.2 *S1P₁R^{-/-}* or *S1P₁R^{+/+}* mice were collected at day 13.5, and used to reconstitute CD45.1 mice. Recipient mice were subjected to whole body irradiation from a [¹³⁷Cs] radiation source (Gamma cell-40, MDS Nordion, Kanata, Ontario, Canada) in two fractions 4 h apart at total doses of 10 Gy. For bone marrow transplant experiments, recipient wild-type mice (WT CD45.1) were reconstituted with 1×10^6 fetal liver cells via the lateral tail vein immediately after whole body irradiation. Engraftments were confirmed by flow cytometry analysis of peripheral blood CD45.1 cell numbers. Ten weeks after engraftment, chimeric mice were given TG peritonitis and peritoneal cell numbers analyzed by flow cytometry.

In vitro macrophage chemotaxis

Chemotaxis assays were performed using a modified Boyden chamber (NeuroProbe, Gaithersburg, MD). BMDM were washed in PBS, and serum starved for 2 h in DMEM. For cAMP studies, cells were pretreated for 30 min prior to migration. Cells were removed from dishes using 5 mM EDTA in PBS and resuspended in DMEM. The 3×10^5 cells were

placed in the top well of the migration chamber and separated from chemoattractants in the bottom well by a filter with 5- μ m pores. Cells were allowed to migrate at 37° C, 5% CO₂ for 2 h, filters were fixed in 4% paraformaldehyde, the top surface wiped clean of nonmigrating cells, and the filters were stained in 0.1% crystal violet. Migration was quantified by eluting the crystal violet in 10% acetic acid and measuring the OD at 495 nm.

Live cell chemotaxis was performed using a Dunn chamber (Hawksley, Lansing, U.K.) (27). BMDM were washed in PBS and starved for 2 h in DMEM. Cells removed from petri dishes using 5 mM EDTA in PBS and resuspended in DMEM with 2% charcoal-stripped FBS and 10 mM HEPES. The 100×10^5 cells were allowed to adhere to precleaned glass coverslips and placed inverted on top of the Dunn chamber. Chemoattractants were placed in the central well and cell chemotaxis was recorded at 37°C, 5% CO₂ for 2 h using an Axiovert 200M 510 Meta confocal microscope (Zeiss, Jena, Germany). Image analysis was performed using MetaMorph (Molecular Devices, Sunnyvale, CA).

Western blot and mRNA analysis

BMDM were grown in 10-cm sterile bacterial dishes, stimulated, washed in PBS, and lysed with RIPA buffer (10 mM Tris-Cl pH 8.0, 0.5 mM EGTA, 1% Triton X-100, 0.1% Na deoxycholate, 0.1% SDS, 140 mM NaCl, 1 mM Na orthovanadate, and 1 mM NaF). Equal amounts of protein were separated by 10% SDS-PAGE and blotted onto a nitrocellulose membrane. Immunoblot analyses were performed using phosphospecific Abs for p42/44 MAPK, AKT (Cell Signaling, Danvers, MA), and α p21-activated kinase (PAK, Santa Cruz Biotechnology, Santa Cruz, CA). mRNA expression analysis, RNA extraction, cDNA synthesis, and real-time PCR analysis were performed as described (7). Briefly, total RNA was extracted from elicited neutrophils using RNA-STAT-60 (Tel-Test, Friendswood, TX) according to the manufacturer's instructions. RNA was treated with DNase I, followed by reverse transcription. Equal amounts of cDNA were run in duplicate SYBR green PCR reactions using an ABI 7900HT sequence detection system (Applied Biosystems, Foster City, CA). Data were analyzed to account for reaction efficiency (28) and results expressed relative to β -actin expression.

Measurement of intracellular Ca²⁺ concentration

Cells were sedimented, resuspended in HEPES-buffered medium, containing 20 mM HEPES (pH 7.4), 103 mM NaCl, 4.8 mM KCl, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 0.5 mM CaCl₂, 25 mM NaHCO₃, 15 mM glucose, and 0.1% BSA (fatty acid-free), and then incubated for 40 min with 5 μ M fura 2-AM. Fluorescence emission at 510 nm after excitation at 340 nm wavelength was measured every 0.1 s using a FluoroLog fluorescent spectrophotometer (HORIBA Jobin Yvon, Edison, NJ) and the amount of [Ca²⁺]_i was estimated from the change in the fluorescence of fura 2-loaded cells as described previously (29).

cAMP measurement

cAMP was measured using a cAMP ELISA according to the manufacturer's instructions (Assay Designs, Ann Arbor, MI). Briefly, BMDM were pre-treated for 10 min with 200 μ M 3-isobutyl-1-methylxanthine in HBSS, stimulated with S1P for 15 min, and cAMP was extracted with HCl.

Statistical analysis

Results are representative of three to five independent experiments, including in vivo experiments comparing independent groups of mice, and in vitro cell cultures obtained from separate mice. Statistical significance was determined by unpaired Student *t* test, or χ^2 test

(where appropriate), using $p < 0.05$ as significant. Statistical results are represented as: * $p < 0.05$; ** $p < 0.01$; and *** $p < 0.001$.

Results

The S1P₂ receptor regulates macrophage recruitment during peritonitis

Macrophages are recruited to sites of inflammation as peripheral blood monocytes, which mature into macrophages in the extravascular space. To examine the possible role of S1P₂R in macrophage recruitment, we analyzed the response of WT (*S1p2r*^{+/+}) and *S1p2r*-null (*S1p2r*^{-/-}) mice during TG-induced peritonitis. As shown in Fig. 1A, CD11b⁺F4/80⁺ macrophages were the major population of cells recruited to the peritoneum 5 d after challenging mice with TG. Many of the recruited macrophages also expressed an intermediate level of the murine DC marker CD11c (Fig. 1A). This CD11b⁺F4/80⁺CD11c^{int} macrophage population was efficiently recruited to the peritoneum in both *S1p2r*^{+/+} and *S1p2r*^{-/-} mice (Fig. 1A). However, on day 5 of peritonitis *S1p2r*^{-/-} mice showed a 44% increase in the absolute number of macrophages when compared with *S1p2r*^{+/+} mice (Fig. 1B). No significant differences in neutrophil and lymphocyte counts were observed (Fig. 1B). Together, these data suggest that S1P₂R restrains macrophage migration into the inflamed peritoneum.

Macrophages recruited to inflamed tissues actively migrate to draining LNs (23), and their egress is an essential part of resolution. This raised the possibility that macrophage numbers in the peritoneum of *S1p2r*^{-/-} mice were increased because of impaired migration to draining LNs. Fifteen days after TG challenge, macrophages remained the major cell type in the peritoneum of *S1p2r*^{+/+} and *S1p2r*^{-/-} mice (Fig. 1A). However, inflammation had resolved, as less macrophages remained in the peritoneum on day 15 when compared with day 5 (Fig. 1B). Loss of S1P₂R did not appear to alter resolution, as equivalent number of macrophages remained in the peritoneum of *S1p2r*^{+/+} and *S1p2r*^{-/-} mice (Fig. 1B). The egress of macrophages in this setting was confirmed by an increase in macrophage content in draining LNs. As shown in Fig. 1C, both *S1p2r*^{+/+} and *S1p2r*^{-/-} mice had equivalent numbers of CD11b⁺F4/80⁺ macrophages in their LNs on day 15. These cells were notably absent in non-draining LNs (Fig. 1C), and were also absent in draining LNs on day 5 (data not shown). To assess the distribution of myeloid cells during peritonitis, we measured peripheral monocyte and splenic macrophage numbers. No significant differences were found between *S1p2r*^{+/+} and *S1p2r*^{-/-} mice (Supplemental Fig. 1). These results suggest that S1P₂R inhibits the recruitment of macrophages into the inflamed peritoneum, whereby loss of S1P₂R results in enhanced macrophage accumulation during peritonitis. In addition, we show that S1P₂R does not influence the egress of macrophages into LNs.

The S1P₂ receptor inhibits macrophage chemotaxis

The enhanced macrophage influx during peritonitis in *S1p2r*-null mice led us to the hypothesis that S1P serves as a negative regulator of migration in the macrophage lineage. To study the effects of S1P in more detail, we sought to use in vitro models of migration. Although monocytes are the initial cell type recruited during inflammation, this cell type is difficult to isolate from mice for in vitro studies, whereas primary BMDM have been used widely to study myeloid cell migration in vitro (30,31). Primary mouse macrophages express mainly S1P₁R and S1P₂R, as determined by real-time RT-PCR (Fig. 2A). This S1P receptor expression profile gives S1P the potential to both stimulate and inhibit the migration of macrophages. To test the effects of S1P on macrophage migration, we generated BMDM and exposed them to chemoattractant gradients using a modified Boyden chamber. S1P was not chemotactic for BMDM over a wide concentration range (1 nM–10 μ M) (Supplemental Fig. 2). As expected, BMDM migrated efficiently to both C5a and

CXCL12 (SDF-1) (Fig. 2B, 2C). When BMDM were exposed to S1P in combination with C5a or CXCL12, S1P inhibited migration toward both chemoattractants (Fig. 2B, 2C). To examine the role of S1P₂R in S1P inhibition of macrophage migration we generated BMDM from *S1p2r* knockout mice (25). Although S1P inhibited migration in *S1p2r*^{+/+} BMDM, S1P was not inhibitory in *S1p2r*^{-/-} cells (Fig. 2B, 2C). These results demonstrate that S1P₂R signaling inhibits chemotaxis in BMDM. They also suggest that loss of S1P₂R in vivo may lead to enhance migration in the macrophage lineage, and may explain the increased accumulation of macrophages during peritonitis (Fig. 1B).

S1P reduces macrophage migration speed but does not alter directionality

To examine the cellular effects of S1P in more detail, we performed live cell chemotaxis assays using a direct-viewing Dunn chemotaxis chamber (27). BMDM were exposed to gradients of C5a, with or without S1P, and migration patterns of individual cells were recorded to determine cell migration speed and directionality, as described in *Materials and Methods*. As shown in Fig. 3A, C5a induced the directional migration of WT BMDM away from their starting position (at X = 0, Y = 0) and up the concentration gradient of C5a (upward on the y-axis). However, when exposed to C5a and S1P together, WT BMDM migrated less, as evident by their clustering around their starting position (at X = 0, Y = 0) (Fig. 3B). These effects were absent in *S1p2r*^{-/-} cells (Fig. 3C, 3D), confirming the inhibitory role of S1P₂R in regulating macrophage migration. We next quantified the effects of S1P on directionality, an important component of efficient chemotaxis. As shown in Fig. 3E and 3F, BMDM migrated up the concentration gradient of C5a (arrows mark the mean direction), and S1P did not alter this directionality. In addition, we found no evidence that S1P alters C5a-induced cell polarization in BMDM. In the presence of a C5a gradient, the percentage of BMDM with lamellipods was unaltered by S1P (data not shown). Likewise, membrane translocation of PH-Akt-cerulean in RAW263.7 cells in response to C5a was unaltered by S1P (data not shown). We also quantified the effects of S1P on migration speed and distance (see *Materials and Methods*). S1P₂R inhibited the average velocity of cells migrating toward C5a (Fig. 3G). This resulted in a corresponding decrease in the average distance that BMDM traveled (Fig. 3H) and also greatly inhibited the ability of BMDM to travel distances of >100 μm (Fig. 3I). These results confirm that S1P, signaling through S1P₂R, inhibits macrophage migration. In addition, they show that S1P inhibits cell migration speed without altering directionality.

S1P₂R selectively inhibits chemoattractant-induced Akt phosphorylation

To determine whether S1P-mediated inhibition of chemotaxis resulted from alterations in chemoattractant receptor signaling, we analyzed the effects of S1P on C5a receptor (C5aR) signaling in BMDM. As shown in Fig. 4A, C5a induced rapid phosphorylation of Akt and p42/44 MAPK. Akt is a serine/threonine kinase whose activity and phosphorylation are controlled by the PI3K product phosphatidylinositol (3,4,5)-trisphosphate (PIP₃). Thus, Akt phosphorylation is one indicator of PIP₃ production. PIP₃ species are vital to cell migration, regulating polarity in eukaryotic cells, including neutrophils and macrophages (32). In BMDM that stimulated simultaneously with S1P and C5a, S1P inhibited Akt phosphorylation, but not the phosphorylation of p42/44 MAPK (Fig. 4A). S1P stimulation also selectively inhibited CXCL12-induced Akt phosphorylation (data not shown). S1P stimulation in *S1p2r*^{-/-} BMDM failed to inhibit C5a-induced Akt phosphorylation (Fig. 4A). S1P₂R has previously been shown to inhibit Akt phosphorylation and inhibit the small GTPase Rac (9,10). Next, we examined the phosphorylation of the PAK, a common mediator of Rac function in leukocytes, including chemotaxis (33,34). As shown in Fig. 4B, C5a-induced PAK phosphorylation was not altered by S1P, at any of the time points tested. Thus, S1P₂R signaling selectively alters chemoattractant-stimulated Akt phosphorylation in

BMDM. These results suggest that S1P₂R disrupts PI3K signaling, leading to inhibition of migration.

Like C5aR, S1P₁R is known to activate Akt and p42/44 MAPK (35). However, only minimal phosphorylation of Akt and p42/44 MAPK was observed with S1P stimulation (Fig. 4A), consistent with the lack of migration observed toward S1P in BMDM (Supplemental Fig. 2A). Thus, we have demonstrated that the S1P₁ receptor is expressed in BMDM at the mRNA level (Fig. 2A), and that the receptor can signal to downstream intermediates, but S1P₂R signaling appears to have greater functional significance in both signaling regulation and migration. In support of the lack of S1P₁R function in macrophage migration, we also failed to detect any differences in peritonitis in chimeric mice lacking hematopoietic S1P₁R (Supplemental Fig. 2B).

Attenuation of chemoattractant receptor signaling can occur as a result of receptor desensitization, where repeated or continuous stimulation of G protein coupled receptors results in diminished responsiveness. Originally identified in β -adrenergic receptors, receptor desensitization can result from sequential stimulation of cells with ligands for the same receptor (homologous desensitization) or with ligands of different receptors (heterologous desensitization) (36). C5aR is known to undergo both homologous and heterologous desensitization (37–39). Because costimulation of macrophages with S1P and C5a, resulted in decreased migration and Akt phosphorylation compared with C5a alone, we considered the possibility that S1P₂R ligation heterologously desensitizes C5aR responsiveness. We measured intracellular Ca²⁺ concentration to examine C5aR signaling in real time. As shown in Fig. 5A, sequential stimulation of macrophages with C5a resulted in a diminished Ca²⁺ response, indicating homologous desensitization of the C5aR. However, S1P stimulation did not inhibit the Ca²⁺ response to C5a, or did S1P induce an increase in intracellular Ca²⁺ (Fig. 5B). The lack of Ca²⁺ response after S1P stimulation is surprising, given that S1P₂R has been shown to couple to G_q and phospholipase C, and to induce Ca²⁺ flux in other cell types (40). Our results indicate that S1P does not desensitize C5aR, and implicate an inhibitory pathway downstream from C5aR.

PTEN is not required for S1P₂R-mediated inhibition

The phosphatase PTEN has been shown to be important for efficient cell migration by localizing and balancing PI3K signaling (41–43). PTEN serves as a negative regulator of the PI3K pathway by dephosphorylating PIP₃ at the 5' position. PTEN has also been postulated to mediate the inhibitory effects of S1P₂R on migration and Akt phosphorylation in endothelial cells and fibroblasts but not in glioblastoma cells (7,8,10). Global deletion of *Pten* is embryonic lethal (44,45). To investigate whether PTEN is required for S1P₂R inhibition in macrophages, we generated inducible *Pten* knockout mice by crossing floxed *Pten* mice with mice expressing inducible *Cre* recombinase (*Pten*^{loxP/loxP}; *CreER*^{T2} mice). As described in *Materials and Methods*, BMDM lacking PTEN (*Pten* Δ/Δ) were generated after *Pten* deletion in vivo. *Pten* Δ/Δ BMDM essentially lacked the PTEN protein, and had a corresponding enhancement of basal Akt phosphorylation (Fig. 6A). *Pten*^{+/+} and *Pten* Δ/Δ BMDM migrated equally well toward C5a, and despite the loss of PTEN, *Pten* Δ/Δ BMDM migration was equally inhibited by S1P (Fig. 6B). In addition, loss of PTEN did not alter the ability of S1P to inhibit Akt phosphorylation. As shown in Fig. 6C, S1P-inhibited C5a induced Akt phosphorylation in both *Pten*^{+/+} and *Pten* Δ/Δ BMDM, whereas p42/44 MAPK phosphorylation remained unaltered by S1P. Our results clearly indicate that PTEN is not required for S1P₂R to inhibit migration in macrophages. Also, lack of PTEN does not alter the ability of S1P₂R to reduce chemoattractant-stimulated Akt phosphorylation. Our results in macrophages confirm the PTEN-independent effect of S1P₂R recently reported in glioma cells (7,8,46).

S1P₂R stimulates cAMP production to inhibit macrophage migration

The second messenger cAMP has been shown to suppress multiple leukocyte functions, inhibiting cytokine production, phagocytosis, and chemotaxis (47–49). cAMP is synthesized from ATP by adenylate cyclase, which is typically activated by ligand binding to G_s coupled receptors (50). More recently, Jiang and colleagues demonstrated a novel mechanism for cAMP production, whereby S1P₂R activates adenylate cyclase by coupling to G_{12/13} (51,52). The actions of cAMP are primarily mediated by two intracellular cAMP effectors: PKA and exchange proteins activated by cAMP (Epac). PKA is a heterotetrameric serine/threonine kinase that phosphorylates a wide range of proteins, to mediate the majority of cAMP effector functions. Epac is a guanine nucleotide exchange factor for the small GTPase Rap (53). Although Epac is less well studied than PKA, it is likely that PKA and Epac can act both alone and in concert to inhibit leukocyte functions (48,54,55).

Because the role of S1P₂R in negatively regulating migration parallels the inhibitory actions of cAMP, we hypothesized that cAMP mediates S1P₂R's inhibition of macrophage migration. We first sought to confirm that S1P induces cAMP production in macrophages. As shown in Fig. 7A, S1P stimulation of WT BMDM led to a significant increase in cAMP. The increase in cAMP was mediated by S1P₂R, as *S1p2r*^{-/-} BMDM did not generate cAMP in response to S1P stimulation (Fig. 7A). The increase in cAMP in BMDM is likely mediated by G_{12/13}, as S1P receptors, including S1P₂R, do not couple to G_s receptors (56). Also, it is likely that S1P₂R increases cAMP by activating adenylate cyclase, and not by inhibiting phosphodiesterases, because the increase in cAMP occurred even in the presence of phosphodiesterase inhibitors, as described in *Materials and Methods*. As shown in Fig. 7B, the cell-permeable cAMP analog 8-Br-cAMP inhibited BMDM migration toward C5a. The inhibitory actions of cAMP on leukocytes have primarily been attributed to the effects of PKA. To better define the pathway of cAMP inhibition, we used cAMP analogs with specificity for activating PKA (6-Bnz-cAMP) and Epac (8-pCPT-2'-O-Me-cAMP) (57,58). The PKA-specific analog 6-Bnz-cAMP exhibited identical inhibition of macrophage migration, whereas the Epac-specific analog 8-pCPT-2'-O-Me-cAMP did not inhibit migration (Fig. 7B). Next, we tested the effects of 8-Br-cAMP on Akt phosphorylation. As shown in Fig. 7C, 8-Br-cAMP treatment of BMDM blocked the ability of C5a to induce Akt phosphorylation, in a similar fashion to S1P. Thus, S1P₂R stimulation causes cAMP generation in macrophages, and elevated levels of intracellular cAMP inhibit macrophage migration and Akt phosphorylation. Taken together, these results clearly suggest that S1P₂R stimulates the production of cAMP to inhibit macrophage migration. In addition our results suggest that PKA is the primary effector for cAMP inhibition of migration in macrophages. To our knowledge, this is the first report examining the effects of PKA and Epac selective cAMP analogs on macrophage migration.

Discussion

The mobilization of leukocytes to sites of inflammation is an essential step in host defense; however, excessive or prolonged influx of effector cells can lead to tissue destruction and dysfunction. With the potential for both benefit and harm, it is likely that leukocyte mobilization is tightly regulated. Although there has been considerable progress in identifying positive regulators of leukocyte recruitment, negative regulators of this process remain poorly understood. In this report, we have identified S1P as a negative regulator of macrophage recruitment during peritonitis. The negative effects of S1P are mediated by S1P₂R, as loss of S1P₂R resulted in increased accumulation of macrophages after TG challenge. This suggests that pharmacologic agonists of S1P₂R may reduce macrophage accumulation at sites of inflammation. These results contrast the known functions of S1P on the trafficking of other leukocytes. The best-studied role for S1P is in lymphocyte recirculation, where S1P₁R is a positive regulator of lymphocyte egress from lymphoid

organs (4). This process is thought to rely on the vascular S1P gradient high levels of S1P in blood and lymph, and low levels of S1P in interstitial fluid (14). Our laboratory has found biochemical data for an S1P gradient between interstitial fluid, plasma, and lymph. We found that plasma contains ~400 nM S1P and lymph ~80 nM S1P (59), whereas peritoneal exudate during peritonitis contained only 20 nM S1P(60). We propose that macrophage recruitment may rely on S1P within the vasculature to dampen the influx of myeloid cells to sites of inflammation. Our results raise the interesting evolutionary concept of opposing roles for S1P in the trafficking of the lymphocyte and macrophage lineages. This also raises the possibility that disrupting the vascular S1P gradient may modulate macrophage trafficking.

Although macrophages can be long-term residents of tissues, they actively migrate to draining LNs during inflammation (23). Their egress is thought to be important in resolution, by clearing debris and potentially harmful effector cells. We examined the effects of S1P₂R on macrophage egress to LNs and found that *S1p2r*-null macrophages still migrated out of the peritoneum to draining LNs. Although S1P₂R did not effect emigration in this setting, S1P may regulate the migratory behavior of macrophages in other settings. For example, macrophage egress from athero-sclerotic plaques has been suggested as a potentially disease modifying event and hence an important therapeutic target (61). In this setting, S1P₂R could function as a retention signal by inhibiting the migration of macrophage foam cells out of plaques.

In an effort to understand the phenotype of *S1P₂R*-null mice during inflammation, we have demonstrated that S1P inhibits macrophage migration in vitro in an S1P₂R-dependent manner. S1P₂R is known to inhibit migration in variety of cell types (6–10) and we have expanded on previous observations of S1P₂R function. Using live cell imaging, we found that S1P inhibits migratory speed, but does not affect the ability of macrophages to sense the direction of a chemoattractant gradient. S1P₂R signaling selectively inhibited Akt phosphorylation, suggesting that S1P₂R alters the PI3K pathway in macrophages. Together, these findings support the previous observation that altering the PI3K pathway in primary macrophages reduces migratory speed but not directionality (62). S1P₂R-mediated inhibition of Akt has previously been thought to rely on degradation of the Akt activator PIP₃ by the inositide phosphatase PTEN (10). Using PTEN-deficient BMDM, we demonstrated that PTEN is dispensable for the effects of S1P on macrophage migration. The lack of involvement of PTEN in macrophages is not surprising given the conflicting reports on the role of PTEN in the migration of other leukocytes. PTEN has been shown to be dispensable for neutrophil and macrophage migration (62). PTEN was also reported to both inhibit (63,64) and stimulate (65) B cell chemotaxis.

Elevated levels of intracellular cAMP were found to inhibit neutrophil and macrophage migration >30 y ago (66–69). This long-standing inhibitory role for cAMP and the identification of S1P₂R-mediated cAMP signaling in macrophage (51,52) led us to examine the role of cAMP in macrophage migration. S1P stimulation of macrophages led to a significant increase in intracellular cAMP, in an S1P₂R-dependent manner. In addition, treatment of macrophages with cell-permeable cAMP analogs mimicked both the inhibitory effect of S1P on migration and the inhibition of C5a-induced Akt phosphorylation. PKA-selective cAMP analogs also demonstrated that the antimigratory effect of cAMP appeared to be mediated by PKA, and not by the alternate cAMP effector Epac. These results highlight the divergent and independent roles for PKA and Epac in cellular responses to cAMP. This raises questions about the way PKA and Epac responses to intracellular cAMP are separated. Like other intracellular mediators, we suspect that cAMP signaling during migration is localized based on the activator of adenylate cyclase. A-kinase anchoring proteins, which are known to localize PKA, are likely to be involved. Of interest to S1P₂R

signaling, G_{12/13} has been shown to interact with PKA and/or a-kinase anchoring protein 110 (70), but the significance of this interaction remains to be determined.

In summary, we have identified S1P as a regulator of macrophage recruitment to sites of inflammation. S1P₂R-mediated inhibition of macrophage migration likely limits excessive macrophage accumulation at inflammatory sites. Thus, S1P₂R represents a potential pharmacologic target to control the damaging effects of excessive macrophage recruitment. In vitro, we have identified cAMP as a novel pathway in the antimigratory signaling of S1P₂R. This raises the possibility that S1P₂R-induced cAMP may modulate macrophage functions in disease states such as atherosclerosis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used in this paper

BMDM	bone marrow-derived macrophage
C5aR	C5a receptor
DC	dendritic cell
Epac	exchange proteins activated by cAMP
LN	lymph node
PAK	p21-activated kinase
PIP₃	phosphatidylinositol (3, 4, 5)-triphosphate
PKA	protein kinase A
PTEN	phosphatase and tensin homolog
S1P	sphingosine 1-phosphate
S1P₁₋₅R	S1P receptor 1–5
TG	thioglycollate
WT	wild-type

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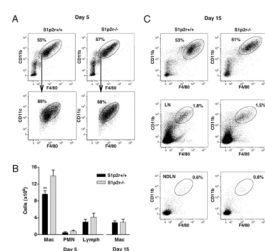
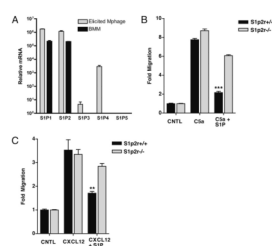


FIGURE 1.

The S1P₂ receptor regulates macrophage recruitment. *S1p2r*^{+/+} and *S1p2r*^{-/-} mice were injected i.p. with TG. Five or 15 d later, cells were collected by peritoneal lavage and analyzed for macrophage content. A, Flow cytometric analysis of peritoneal cells on days 5 and 15, showing percentage of cells that are CD11b⁺F480⁺, and CD11c expression in this population; (day 5 *S1p2r*^{+/+} *n* = 14, *S1p2r*^{-/-} *n* = 11) (day 15 *S1p2r*^{+/+} *n* = 10, *S1p2r*^{-/-} *n* = 9). B, Absolute numbers of peritoneal macrophages, polymorphonuclear neutrophils, and lymphocytes recovered from the peritoneum on day 5 and 15. C, Flow cytometric analysis of draining LNs and non-draining LNs on day 15, showing percentage of cells that are CD11b⁺F480⁺.

**FIGURE 2.**

The S1P₂ receptor inhibits macrophage chemotaxis. *A*, Real-time PCR analysis of macrophage S1P receptor expression. *B* and *C*, In vitro chemotaxis of BMDM analyzed using a modified Boyden chamber. Migration of *Slp2r*^{+/+} and *Slp2r*^{-/-} BMDM toward 10 nM C5a (*B*) or 10 nM CXCL12 (*C*), with or without 10 nM S1P.

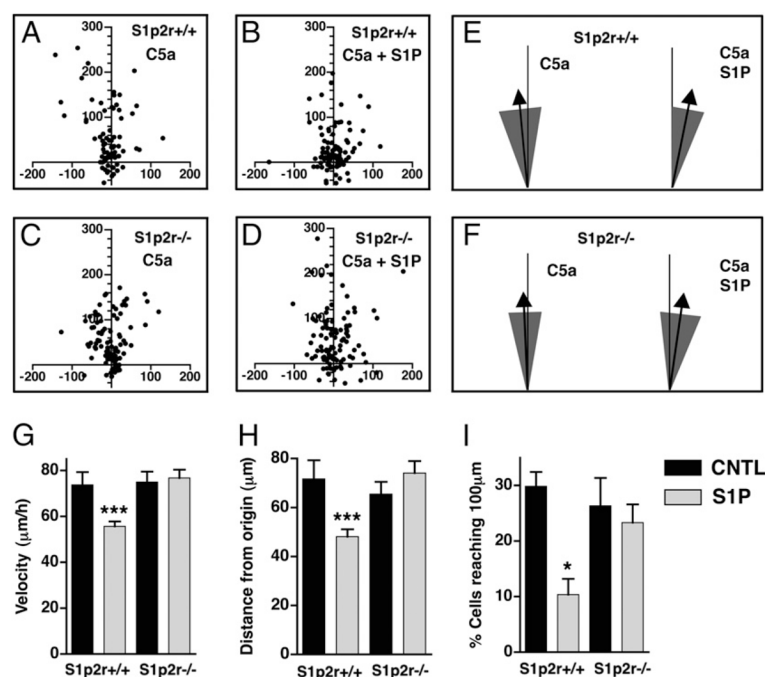
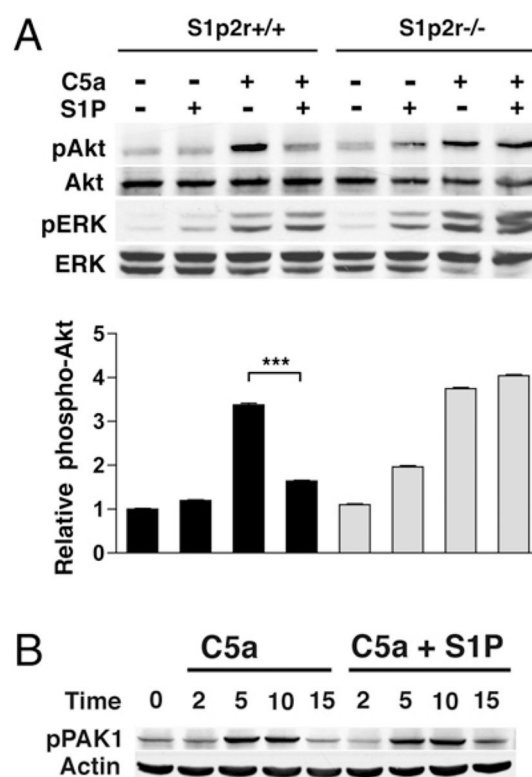


FIGURE 3.

S1P reduces macrophage migration speed but does not alter directionality. A Dunn chamber was used to analyze live cell chemotaxis of *S1p2r^{+/+}* and *S1p2r^{-/-}* BMDM migrating toward 10 nM C5a, with or without 10 nM S1P. A–D, Scatter plots indicating final cell positions. E and F, Direction of migration of *S1p2r^{+/+}* (E) or *S1p2r^{-/-}* (F) BMDM, represented as mean direction (arrow) and 95% CI (shaded area). G–I, Quantification of velocity (G), distance from origin (H), and the percentage of cells reaching 100 μm from origin (I).

**FIGURE 4.**

S1P₂R selectively inhibits chemoattractant-induced Akt phosphorylation. *S1p2r^{+/+}* and *S1p2r^{-/-}* BMDM were analyzed by Western blot for protein phosphorylation. **A**, Phospho-Akt and phospho-ERK analysis after 5-min stimulation with 10 nM C5a, with or without 100 nM S1P. **B**, Time-course of phospho-PAK1 analysis in *S1p2r^{+/+}* BMDM stimulated with 10 nM C5a, with or without 100 nM S1P.

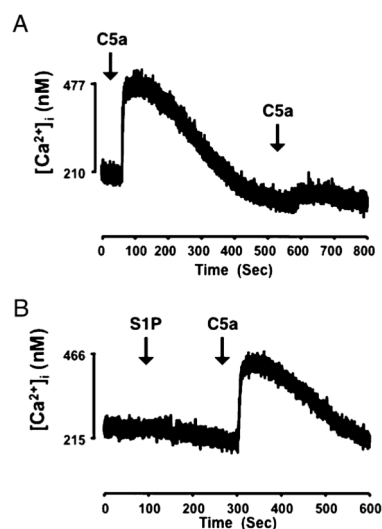


FIGURE 5.

S1P₂R does not desensitize the C5aR. Intracellular calcium was measured in cell suspensions of WT macrophages loaded with fura 2, as described in *Materials and Methods*. Cells were sequentially stimulated with 1 nM C5a (A) or stimulated with 1 μ M S1P, followed by 1 nM C5a (B), and intracellular calcium content measured in real time.

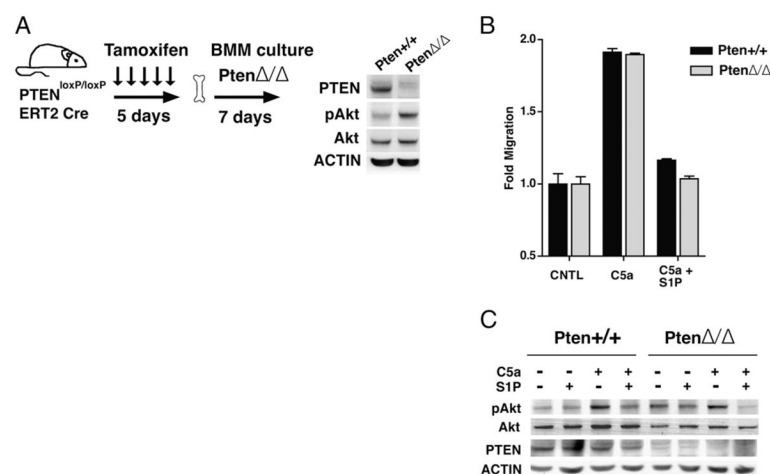
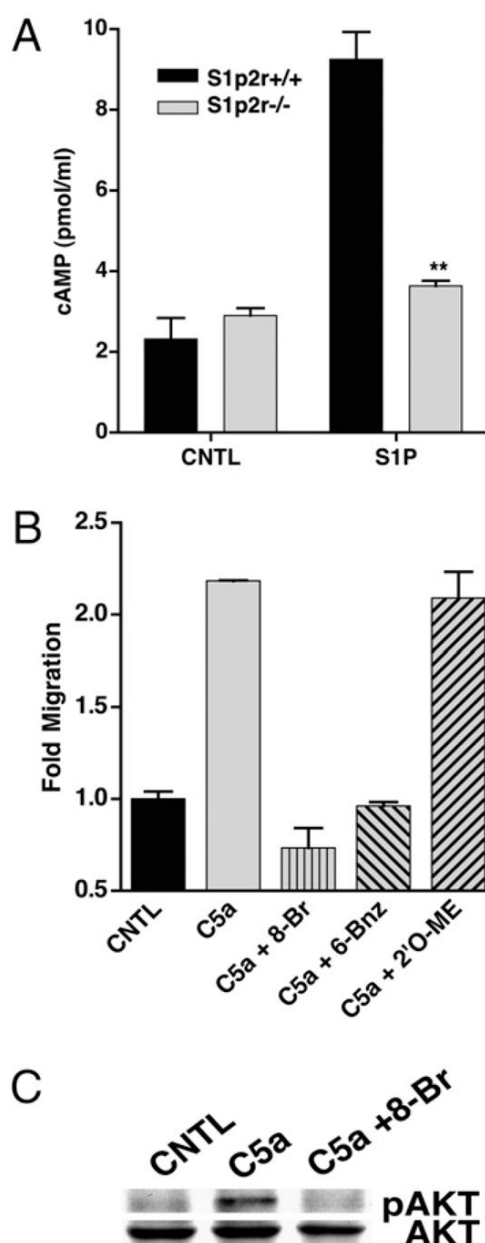


FIGURE 6.

PTEN is not required for S1P₂R-mediated inhibition. *A*, *Pten*^{loxP/loxP}, *Cre*^{ERT2}, and *Pten*^{loxP/loxP} mice were fed tamoxifen for 5 d and bone marrow was cultured to generate *Pten*^{Δ/Δ} and *Pten*^{+/+} BMDM, respectively. *Inset* shows immunoblot analysis of BMDM extracts for respective Abs. *B*, In vitro migration of *Pten*^{Δ/Δ} and *Pten*^{+/+} BMDM toward 10 nM C5a, with or without 10 nM S1P, was measured using a modified Boyden chamber. *C*, Western blot analysis of phospho-Akt in *Pten*^{Δ/Δ} and *Pten*^{+/+} BMDM stimulated with 10 nM C5a, with or without 100 nM S1P.

**FIGURE 7.**

*S1P*₂R stimulates cAMP production to inhibit BMDM migration. **A**, Intracellular cAMP levels were measured by ELISA in *S1p2r*^{+/+} and *S1p2r*^{-/-} BMDM after stimulation with 10 nM S1P. **B**, BMDM were pretreated for 30 min with 500 μ M cAMP analogs and then migrated toward 10 nM C5a in a modified Boyden chamber, in the presence of cAMP analogs. **C**, Western blot analysis of phospho-Akt in BMDM pretreated with 500 μ M 8-Br-cAMP for 30 min.