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

Jason Michaud, Dong-Soon Im and Timothy Hla

*J Immunol* published online 30 December 2009
http://www.jimmunol.org/content/early/2009/12/30/jimmunol.0901586

Supplementary Material
http://www.jimmunol.org/content/suppl/2009/12/30/jimmunol.0901586.DC1

Why *The JI*?
- **Rapid Reviews!** 30 days* from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Speedy Publication!** 4 weeks from acceptance to publication

*average

Subscription
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Inhibitory Role of Sphingosine 1-Phosphate Receptor 2 in Macrophage Recruitment during Inflammation

Jason Michaud,* Dong-Soon Im,*† and Timothy Hla*,‡

Macrophage recruitment to sites of inflammation is an essential step in host defense. However, the mechanisms preventing excessive accumulation of macrophages remain largely 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, S1P2R 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 S1P1R 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. The Journal of Immunology, 2010, 184: 000–000.

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 S1P1–5R]). 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 G1-coupled receptor that stimulates migration in endothelial cells and lymphocytes (4, 5). In contrast, S1P2R is a G12/13-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 S1P1R-mediated 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 chemotactants 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
Sphingosine 1-phosphate and macrophage migration

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 1 mL PBS. The 3 \times 10^5 cells were placed in the bottom well of the migration chamber and separated from chemotacticants in the bottom well by a filter with 5-\mu m pores. Cells were allowed to migrate at 37\(^\circ\)C, 5% CO\(_2\) 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, Lancing, 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 \times 10^6 cells were allowed to adhere to pre-cleaned glass covershells and placed inverted on top of the Dunn chamber. Chemotacticants were placed in the central well and cell chemotaxis was recorded at 37\(^\circ\)C, 5% CO\(_2\) 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 EDTA, 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 phospho-specific Abs for p42/44 MAPK, AKT (Cell Signaling, Danvers, MA), and \(\alpha\) p21-activated kinase (P3K, 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 \(\beta\)-actin expression.

Measurement of intracellular Ca\(^{2+}\) 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 KH\(_2 PO_4\), 1.2 mM MgSO\(_4\), 0.5 mM CaCl\(_2\), 25 mM NaHCO\(_3\), 15 mM glucose, and 0.1% BSA (fatty acid-free), and then incubated for 40 min with 5 \mu 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 \([\text{Ca}^{2+}]\) 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 \mu 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’s t test, or \(\chi^2\) 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 S1P\(_2\) 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 S1P\(_2\)R in macrophage


ingestion and S1p2r knockout mice, we show that S1P-R negatively regulates the accumulation of macrophage during inflammation. In vitro, we show that S1P inhibits macrophage migration through S1P-R ligation. S1P-R signaling stimulates cAMP production and inhibits chemotactant-induced Akt phosphorylation. However, chemotactant-induced receptor signaling remains intact, including calcium flux and MAPK phosphorylation. In addition, protein kinase A (PKA)-selective cAMP analogs mimic the effects of S1P-R signaling, inhibiting migration, and Akt phosphorylation. Our results demonstrate that S1P-R can influence macrophage recruitment during inflammation, and we identify a novel mechanism for S1P-R in regulating leukocyte migration in vivo.

Materials and Methods

Mice

S1p2r\(^{-/-}\) mice were obtained from Dr. Richard Proia (National Institutes of Health, Bethesda, MD). PtenloxP/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 obtained 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 CsA 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 using CO\(_2\), peritoneum lavaged with 5 ml cold HBSS, and draining peritoneal space. To examine the possible role of S1P2R in macrophage recruitment during inflammation and S1P-R signaling, CD11b and F4/80 expression. To generate PTEN-deficient macrophages, PtenloxP/loxP bone marrow-derived macrophages (BMDM), PtenloxP/loxP; Rosa26 CreER\(^{2}\), and PtenloxP/loxP; Rosa26 CreER\(^{2}\) 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 S1P-R deficiency had no effect on the efficiency of BMDM generation, which typically yielded \(\sim 95\%\) CD11b+ F4/80+ cells.

Peritonitis in S1P-R\(^{-/-}\) fetal liver chimeras

Livers from CD45.2 S1P-R\(^{-/-}\) or S1P-R\(^{+/+}\) mice were collected at day 13.5, and used to reconstitute CD45.1 mice. Recipient mice were subjected to whole body irradiation from a \(^{137}\)Cs source (GammaCell-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 \times 10\(^5\) 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.
recruitment, we analyzed the response of WT (Slp2r+/+) and Slp2r-null (Slp2r−/−) 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^hi^ macrophage population was efficiently recruited to the peritoneum in both Slp2r^+/+ and Slp2r^−/− mice (Fig. 1A). However, on day 5 of peritonitis Slp2r^+/+ mice showed a 44% increase in the absolute number of macrophages when compared with Slp2r^−/− mice (Fig. 1B). No significant differences in neutrophil and lymphocyte counts were observed (Fig. 1B). Together, these data suggest that Slp2R 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 macrophages numbers in the peritoneum of Slp2r^−/− 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 Slp2r^+/+ and Slp2r^−/− 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 Slp2R did not appear to alter resolution, as equivalent number of macrophages remained in the peritoneum of Slp2r^+/+ and Slp2r^−/− 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 Slp2r^+/+ and Slp2r^−/− 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 Slp2r^+/+ and Slp2r^−/− mice (Supplemental Fig. 1). These results suggest that Slp2R inhibits the recruitment of macrophages into the inflamed peritoneum, whereby loss of Slp2R results in enhanced macrophage accumulation during peritonitis. In addition, we show that Slp2R does not influence the egress of macrophages into LNs.

The Slp2 receptor inhibits macrophage chemotaxis

The enhanced macrophage influx during peritonitis in Slp2r-null mice led us to the hypothesis that Slp serves as a negative regulator of migration in the macrophage lineage. To study the effects of Slp 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 Slp2R and Slp3R, as determined by real-time RT-PCR (Fig. 2A). This Slp receptor expression profile gives Slp the potential to both stimulate and inhibit the migration of macrophages. To test the effects of Slp on macrophage migration, we generated BMDM and exposed them to chemotactrant gradients using a modified Boyden chamber. Slp was not chemotactic for BMDM over a wide concentration range (1 nM–10 μM) (Supplemental Fig. 2). As expected, BMDM migrated efficiently to both C5a and CXCL12 (SDF-1) (Fig. 2B, 2C). When BMDM were exposed to Slp in combination with C5a or CXCL12, Slp inhibited migration toward both chemoattractants (Fig. 2B, 2C). To examine the role of Slp2R in Slps inhibition of macrophage migration we generated BMDM from Slp2R−/− knockout mice (25). Although Slp inhibited migration in Slp2r^+/+ BMDM, Slp was not inhibitory in Slp2r^−/− cells (Fig. 2B, 2C). These results demonstrate that Slp2R signaling inhibits chemotaxis in BMDM. They also suggest that loss of Slp2R in vivo may lead to enhance migration in the macrophage lineage, and may explain the increased accumulation of macrophages during peritonitis (Fig. 1B).
SIP reduces macrophage migration speed but does not alter directionality

To examine the cellular effects of SIP 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 SIP, 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 SIP 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 SIP 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 SIP did not alter this directionality. In addition, we found no evidence that SIP alters C5a-induced cell polarization in BMDM. In the presence of a C5a gradient, the percentage of BMDM with lamellipods was unaltered by SIP (data not shown). Likewise, membrane translocation of PH-Akt-creulane in RAW263.7 cells in response to C5a was unaltered by SIP (data not shown). We also quantified the effects of SIP on migration speed and distance (see Materials and Methods). S1P-R 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 SIP, signaling through S1P-R, inhibits macrophage migration. In addition, they show that SIP inhibits cell migration speed without altering directionality.

SIP2R selectively inhibits chemoattractant-induced Akt phosphorylation

To determine whether SIP-mediated inhibition of chemotaxis resulted from alterations in chemoattractant receptor signaling, we analyzed the effects of SIP 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 SIP 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). S1P-R 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, S1P-R signaling selectively alters chemoattractant-stimulated Akt phosphorylation in BMDM. These results suggest that S1P-R 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 SIP stimulation (Fig. 4A), consistent with the lack of migration observed toward SIP 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 S1P-R signaling appears to have greater functional significance in

FIGURE 2. The SIP2 receptor inhibits macrophage chemotaxis. A, Real-time PCR analysis of macrophage SIP receptor expression. B and C, In vitro chemotaxis of BMDM analyzed using a modified Boyden chamber. Migration of S1p2+/+ and S1p2r−/− BMDM toward 10 nM C5a (B) or 10 nM CXCL12 (C), with or without 10 nM SIP.
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 Gq 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 PI3K 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 (PtenLoxP/LoxP;CreERT2 mice). As described in Materials and Methods, BMDM lacking PTEN (PtenΔΔ) were generated after Pten deletion in vivo. PtenΔΔ BMDM essentially lacked the PTEN protein, and had a corresponding enhancement of basal Akt phosphorylation (Fig. 6A). PtenΔΔ and PtenΔΔ BMDM migrated equally well toward C5a, and despite the loss of PTEN, PtenΔΔ 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 PtenΔΔ and PtenΔΔ BMDM,

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.
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 S1P 2R 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 Gs coupled receptors (50). More recently, Jiang and colleagues demonstrated a novel mechanism for cAMP production, whereby S1P1R activates adenylate cyclase by coupling to G12/13 (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 S1P2Rs 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 S1p2r2/2 BMDM did not generate cAMP in response to S1P stimulation (Fig. 7A). The increase in cAMP in BMDM is likely mediated by G12/13, as S1P receptors, including S1P 2R, do not couple to Gs 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).

**FIGURE 6.** PTEN is not required for S1P2R-mediated inhibition. A, PtenloxP/loxP Cre ERT2, and PtenloxP/loxP mice were fed tamoxifen for 5 d and bone marrow was cultured to generate PtenΔ/Δ and Pten++ BMDM, respectively. Inset shows immunoblot analysis of BMDM extracts for respective Abs. B, In vitro migration of PtenΔ/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 PtenΔ/Δ and Pten++ BMDM stimulated with 10 nM C5a, with or without 100 nM S1P.
The PKA-specific analog 6-Bnz-cAMP exhibited identical inhibitory effects 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, S1P-R 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 S1P-R 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 S1P-R 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 S1P-R on macrophage egress to LNs and found that S1p2r-null macrophages still migrated out of the peritoneum to draining LNs. Although S1P-R did not effect emigration in this setting, S1P may regulate the migratory behavior of macrophages in other settings. For example, macrophage egress from atherosclerotic plaques has been suggested as a potentially disease modifying event and hence an important therapeutic target (61). In this setting, S1P-R 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 S1P-R-null mice during inflammation, we have demonstrated that S1P inhibits macrophage migration in vitro in an S1P-R-dependent manner. S1P-R is known to inhibit migration in variety of cell types (6–10) and we have expanded on previous observations of S1P-R 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 chemotactant gradient. S1P-R signaling selectively inhibited Akt phosphorylation, suggesting that S1P-R 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). S1P-R-mediated inhibition of Akt has previously been thought to rely on degradation of the Akt activator PIP3 by the inositol 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 S1P-R-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 S1P 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 S1P. 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 adenylyl cyclase. A-kinaseanchoring proteins, which are known to localize PKA, are likely to be involved. Of interest to S1P-R signaling, G12/13 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. S1P-R-mediated inhibition of macrophage migration likely limits excessive macrophage accumulation at inflammatory sites. Thus, S1P-R 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 S1P-R. This raises the possibility that S1P-R-induced cAMP may modulate macrophage functions in disease states such as atherosclerosis.

Acknowledgments

We thank Richard Proia for S1P-R-null mice, Dr. Zhihai Li for advice in culturing BMDM, Dr. Guo-Hua Fong for Rosa26 CreER T2 mice, Dr. Kim-berly Dodge-Kafka for helpful suggestions on cAMP signaling, Dr. James Waris for PH-Akt-creuline constructs, and the Center for Cell Analysis and Modeling at the University of Connecticut for help with microscopy. J. M. also thanks the University of Connecticut M.D./Ph.D. Program.

Disclosures

The authors have no financial conflicts of interest.

References


Supplemental Figure 1. A and B, Peripheral blood leukocyte and splenic macrophage content, respectively, in S1P2R−/− and S1P2R+/+ mice at day 5 of thioglycollate peritonitis. C and D, Monocyte, neutrophil and lymphocyte numbers in the peritoneum of S1P2R−/− and S1P2R+/+ mice at 24 hours (C) and 5 days (D) of thioglycollate peritonitis. No significant differences were found.
Supplemental Figure 2. A, S1P is not chemotactic for BMM over a wide concentration range. Migration towards C5α is shown for reference. B, Macrophage cell numbers in the peritoneum of S1P₁R−/− and S1P₁R+/+ bone marrow chimeras at day 5 of thioglycollate peritonitis. No significant differences were found.