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Sphingosine-1-Phosphate Induces an Antiinflammatory Phenotype in Macrophages

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Abstract

Activated macrophages acquire a proinflammatory (classic) or antiinflammatory (alternative) phenotype that influences atherosclerosis. The present study investigated whether sphingosine-1-phosphate (S1P), with its known antiinflammatory effects, could regulate the inflammatory phenotype of lipopolysaccharide (LPS)-stimulated mouse macrophages. Activation of macrophages by LPS significantly increases proinflammatory cytokine secretion. Pretreatment of macrophages with 500 nmol/L S1P markedly reduced LPS-mediated secretion of tumor necrosis factor- α , monocyte chemoattractant protein-1, and interleukin-12. Such antiinflammatory actions were also evident in LPS-stimulated macrophages treated with the S1P1 receptor-specific agonist SEW2871. Pharmacological antagonism of the S1P1 receptor on macrophages using the S1P1-specific antagonist VPC44116 also blocked proinflammatory cytokine secretion in response to LPS. Studies using bone marrow-derived macrophages from S1P2-deficient mice revealed that the S1P2 receptor did not play a pivotal role in this process. Thus, activation of the S1P1 receptor in mouse macrophages limits the expression of proinflammatory cytokines. Furthermore, we demonstrated that S1P increased arginase I activity and inhibited LPS-induced inducible NO synthase activity in LPS-treated macrophages, again through S1P1 receptor activation on macrophages. Analysis of a 1.7-kb region of the murine inducible NO synthase promoter revealed the presence of putative nuclear factor κ B, activator protein-1, and STAT-1 response elements. Using inducible NO synthase promoter-reporter constructs, we found that S1P significantly reduced the nuclear factor κ B-mediated induction of inducible NO synthase. These findings demonstrate an important role for S1P in the regulation of macrophage phenotypic switching. Therefore, we conclude that S1P promotes the production of an alternative antiinflammatory macrophage phenotype through activation of the macrophage S1P1 receptor.

Keywords

macrophage; sphingosine-1-phosphate; arginase I; iNOS; NF κ B; inflammation

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Disclosures

None.

Atherosclerosis is a chronic inflammatory disease.¹ Monocytes/macrophages play key roles in the initiation and progression of atherosclerosis, and can alter their phenotype in response to changes in the local cytokine environment.² Macrophages can be distinctly activated to either a classically activated, or M1, phenotype by proinflammatory molecules such as interferon- γ and lipopolysaccharide (LPS) or to an “alternatively activated,” or M2, phenotype by Th-2 antiinflammatory cytokines such as interleukin (IL)-4.³ Macrophages can also display a M2b activated phenotype when stimulated by immune complexes and LPS.⁴ The M1 and M2 phenotypes are specifically distinguished by the cytokines produced and by the way in which arginine is processed, whereas the M2b phenotype is mainly distinguished by high IL-10 and low IL-12 levels. In classically activated macrophages, Th-1 cytokines trigger the induction of inducible NO synthase (iNOS), causing the production of NO. However, in alternatively activated macrophages, Th-2 cytokines induce arginase (Arg) I, which converts arginine to ornithine and urea.^{5,6} Several studies have shown that on macrophage activation by Th-2 cytokines, the upregulation of Arg activity results in the depletion of arginine and subsequent inhibition of nitric oxide production.^{7,8} However, the exact functional properties of classically and alternatively activated macrophages in vivo remain unclear. Further understanding of the mechanisms contributing to the generation of these macrophage phenotypes and their functions in vivo will provide important information regarding the development of novel targeted therapies for treatment of inflammatory diseases, including atherosclerosis.

Sphingosine-1-phosphate (S1P), a biologically active sphingolipid, plays important roles in the regulation of a variety of cellular processes, including cell survival and vascular maturation, by binding to a family of G protein-coupled receptors (termed S1P₁₋₅).⁹ FTY720, a synthetic analogue of sphingosine and a potent agonist of 4 of 5 S1P receptors (S1P₁, S1P₃, S1P₄, and S1P₅), has been shown to modulate lymphocyte trafficking in mice through activation of the S1P₁ receptor.¹⁰ Ogawa and colleagues reported that the novel S1P₁ receptor agonist KRP-203 reduced experimental autoimmune myocarditis in rats.¹¹ Klingenberg et al recently reported that FTY720 modulated lymphocyte distribution in apolipoprotein E-deficient mice.¹² Major unresolved questions in the field of atherosclerosis are whether S1P promotes antiinflammatory responses in macrophages by interacting with specific macrophage S1P receptors and, thus, whether specific S1P receptors significantly influence atherosclerosis development. Recent studies show an important role for S1P receptors in macrophage function in atherogenesis. Recently, Nofer et al showed that FTY720 can modulate macrophage activation and reduce atherosclerosis development in low-density lipoprotein receptor-deficient mice.¹³ Keul et al similarly reported reduced atherosclerosis in apolipoprotein E-deficient mice treated with FTY720 through modulation of monocyte chemotaxis.¹⁴ Additionally, Brune and colleagues have demonstrated that the production of S1P by sphingosine kinase protects macrophages from apoptosis and triggers macrophage production of the antiinflammatory cytokine transforming growth factor- β .¹⁵ Thus, S1P can regulate survival pathways as well as promote antiinflammatory signals in macrophages, which are important for atherogenesis.

In the present study, we report that during acute inflammation, S1P switches the phenotype of macrophages from a proinflammatory to an antiinflammatory phenotype. We also provide evidence that this switch to an antiinflammatory phenotype is regulated by the S1P₁ receptor. Furthermore, we identify that S1P regulates Arg I and iNOS expression and that regulation of macrophage iNOS expression by S1P is mediated primarily through inhibition of nuclear factor (NF) κ B.

Materials and Methods

An expanded Materials and Methods section is available in the online data supplement at <http://circres.ahajournals.org>.

Mice

Ten-week-old C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, Me). S1P2 receptor-deficient mice on a C57BL/6 background were generated by the laboratory or R.L.P. (NIH).¹⁶ These S1P2-deficient mice have been backcrossed for 7 generations onto C57BL/6J. Wild-type littermates from the colony were used as controls for all studies using S1P2-deficient mice. All experiments followed University of Virginia Animal Care and Use Committee guidelines, and approval for use of rodents was obtained from the University of Virginia.

Isolation of Mouse Peritoneal Macrophages

Eight- to 10-week-old C57BL/6 mice were injected with 2 mL of 3% thioglycollate medium. On day 5 postinjection, mice were anesthetized with isoflurane and injected IP with 5 mL cold PBS with 10 mmol/L EDTA. The PBS was then removed with a syringe, and the process was repeated 3 times. Macrophages from each mouse were plated separately in RPMI/10% FBS overnight. The next day, nonadherent cells were removed by aspiration, and the remaining macrophages were washed thoroughly with PBS and used for experiments.

Isolation of Bone Marrow-Derived Macrophages

Cells were obtained from the tibia and femur bone marrow of wild-type littermates and S1P2 receptor-deficient mice (age, 8 to 10 weeks) and were cultured in the presence of L-929 conditioned medium as described in detail previously.¹⁷

Cell Culture Studies

Macrophages were maintained in RPMI medium 1640/10% FBS as described above. For studies, LPS was added at 10 ng/mL in the absence or presence of 500 nmol/L S1P, 1 μ mol/L SEW2871, 10 μ mol/L VPC44116, 100 ng/mL pertussis toxin, or BAY11-7085 (5 to 10 μ mol/L) as described in each figure legend.

Promoter Cloning and Site-Directed Mutagenesis

The 1749-bp fragment of the 5'-flanking region of the murine iNOS gene was subcloned into the *Kpn*I site of pGL3-Basic. The presence of the iNOS promoter was confirmed by PCR using oligonucleotides specific for regions within the promoter. Mutations in the distal NF κ B site were generated in the piNOS-luc construct by using the Quik-change site-directed mutagenesis kit (Stratagene, La Jolla, Calif) using the following oligonucleotides: 5'-TAA CTT GCA CAC CCA ACT AAA AAA AAC TTT GGG AAC A-3' and 3'-ATT GAA CGT GTG GGT TGA TTT TTT TTT TTG AAA CCC TTG T-5'.

Results

S1P Receptor Expression in Mouse Macrophages

We isolated bone marrow derived macrophages from C57Bl/6J (B6) mice and measured the relative mRNA expression of each S1P receptor using RT-PCR. S1P1 and S1P2 mRNA were expressed in bone marrow-derived macrophages; however, we found no expression of S1P3, S1P4, and S1P5 mRNA (Figure 1) in either B6 or S1P2 knockout (KO) macrophages. We have confirmed similar S1P receptor expression in peritoneal macrophages (data not shown). Furthermore, we did not observe any compensatory upregulation of S1P1 receptor expression in the S1P2 KO macrophages (Figure 1).

S1P Regulation of LPS-Stimulated, Proinflammatory Cytokine Production by Primary Murine Macrophages

LPS has been shown to downregulate Arg I and upregulate iNOS expression.^{18,19} Increased Arg I activity is associated with downregulation of the inflammatory response, whereas iNOS activity is associated with a proinflammatory macrophage response.²⁰ In the present study, we examined whether S1P could modulate the effects of LPS on macrophage activation. S1P is present in the range of 300 nmol/L to 1 μ mol/L in plasma; therefore, we first performed a dose curve of S1P on mRNA expression of Arg I and iNOS in B6 macrophages.²¹ B6 peritoneal macrophages were incubated for 4 hours with 10 ng/mL LPS in the absence or presence of S1P. As shown in Figure 2A, S1P effectively increased Arg I expression and reduced iNOS expression at 100 and 500 nmol/L concentrations of S1P. Because we observed maximal effects using 500 nmol/L S1P, we chose the 500 nmol/L concentration for our experiments. We then examined levels of cytokine mRNA in response to LPS and 500 nmol/L S1P. In the absence of LPS stimulation, no cytokine expression could be detected in macrophages (Figure 2B). Treatment of macrophages with S1P suppressed LPS-induced tumor necrosis factor (TNF) α , monocyte chemoattractant protein-1, IL-12, cyclooxygenase-2, and macrophage inflammatory protein-2 mRNA expression by 3-fold, 2-fold, 6-fold, 5-fold, and 7-fold, respectively ($P < 0.001$; Figure 2B). However, S1P had no effect on the LPS-mediated induction of KC mRNA (Figure 2B). We also measured TNF α secretion by macrophages using ELISA (Figure 2C). Naïve, unstimulated macrophages secreted very little TNF α ; however, incubation of macrophages with 10 ng/mL LPS induced TNF α secretion by 3-fold. S1P reduced TNF α secretion by 30%. Surprisingly, we observed a small, yet significant, increase in TNF α mRNA expression in the presence of S1P alone (Figure 2B), but this did not translate into detectable TNF α protein secretion (Figure 2C). Thus, within the 4-hour time frame of our study, S1P significantly reduced LPS-mediated TNF α secretion. We also observed that S1P reduced interferon- γ -mediated increases in TNF α , as well as oxidized low-density lipoprotein-mediated increases in TNF α and CD36 (data not shown), indicating that the S1P-mediated phenotypic switching of macrophages is not restricted to LPS.

Sphingosine-1-Phosphate Regulates Arg I and iNOS Expression in Macrophages

Given that iNOS and Arg I use the common substrate arginine, for which they compete, and because we observed changes in enzyme expression in response to S1P (Figure 2A), we investigated in more detail the effect of S1P on Arg I and iNOS in B6 macrophages stimulated with LPS. First, we examined the potential regulation of different Arg isoforms by S1P. The addition of LPS to B6 macrophages inhibited Arg I mRNA expression (Figure 2D). Incubation of macrophages with S1P restored Arg I mRNA expression to control levels (Figure 2D). In contrast, neither LPS nor S1P affected Arg-2 mRNA expression. Furthermore, we found that S1P significantly reduced LPS-mediated iNOS mRNA expression in primary macrophages (Figure 2D).

We next measured Arg I and iNOS enzyme activities. In wild-type macrophages, LPS suppressed Arg I activity, and both S1P and SEW stimulated Arg I activity, as measured by macrophage urea production (see black bars in Figure 3A). These results indicate that Arg I is indeed responsive to the S1P pathway in macrophages. Furthermore, in agreement with our iNOS mRNA results, we observed a significant decrease in LPS-induced iNOS enzymatic activity in macrophages on stimulation with S1P (see black bars in Figure 3B). Collectively, these data strongly suggest that S1P shifts nitrogen use away from the iNOS pathway toward the Arg I pathway. Thus, we hypothesize that S1P acts in macrophages, at least in part, by a dual mechanism of both suppressing the induction of iNOS and enhancing Arg I expression.

Identification of S1P1 As an Antiinflammatory Receptor in Macrophages

We next wished to identify the specific receptor target that mediates the antiinflammatory S1P response in macrophages. As shown in Figure 1, macrophages express S1P1 and S1P2 receptors. Mice that are deficient in the S1P1 receptor show embryonic lethality attributable to the critical role that S1P1 plays in vascular maturation.²² Thus, we used pharmacological reagents that are selective for S1P1. SEW2871 [5-(4-Phenyl-5-trifluoromethylthiophen-2-yl)-3-(3-(3-trifluoromethylphenyl)-1,2,4-oxadiazole)] is a selective S1P1 receptor agonist that is 30-fold less potent than S1P at S1P1, with no agonist activity at S1P2 at concentrations up to 10 micromolar.²³ VPC44116, an *N*-arylamide phosphonate, is a selective S1P1 receptor antagonist that exhibits a K_i of 30 nmol/L for S1P1.²⁴ VPC44116 has recently been shown to prevent the protective effects of FTY720 on acute renal injury in mice.²⁵ We found a significant reduction in iNOS activity (see black bars in Figure 3B) and TNF α secretion (see black bars in Figure 3C) in wild-type macrophages treated with SEW2871 compared to LPS. We observed a significant increase in Arg I activity in SEW2871-treated macrophages (see black bars in Figure 3A). The activation of S1P1 by SEW2871 paralleled the S1P-mediated changes in macrophage activation in all cases, suggesting that the S1P1 receptor mediates the antiinflammatory effects of S1P in macrophages.

To confirm the role of the S1P1 receptor, we used bone marrow–derived macrophages from S1P2-deficient mice (see red bars in Figure 3).¹⁶ These mice are viable, but have been found recently to be deaf.¹⁶ We confirmed the absence of S1P2 expression in these macrophages; also, there was no compensatory upregulation of S1P1 in these cells (Figure 1). In S1P2-deficient macrophages, LPS caused a slightly blunted response in TNF α secretion (red bars in Figure 3C) and iNOS activity (red bars in Figure 3B), suggesting that the absence of the S1P2 receptor impacts the magnitude of the LPS response in macrophages. Arg I activity, however, appeared similar in both wild-type and S1P2-deficient macrophages (Figure 3A). Both S1P and SEW2871 again significantly reduced LPS-induced iNOS activity and TNF α secretion by these S1P2-deficient macrophages (red bars in Figure 3). S1P and SEW2871 also increased Arg I activity in both B6 and S1P2 KO macrophages to similar extents (Figure 3).

We also found that VPC44116, a selective S1P1 receptor antagonist, was a potent inhibitor of both S1P and SEW2871 action in wild-type and S1P2KO macrophages (see VPC bars in Figure 3). VPC44116 worked as effectively in S1P2KO macrophages as in wild-type macrophages to prevent S1P and SEW2871 action on TNF α secretion as well as iNOS and Arg I activities (see red bars in Figure 3).

S1P1 is solely coupled to $G_{\alpha i}$ whereas S1P2 couples primarily to $G_{\alpha q}$. To uncouple $G_{\alpha i}$ signaling in macrophages, we treated macrophages overnight with 100 ng/mL pertussis toxin as another means to target S1P1. As shown in Figure 4, treatment of macrophages with pertussis toxin reduced the action of both S1P and SEW2871 on Arg I and iNOS activities (blue bars in Figure 4A and 4B), as well as TNF α secretion (blue bars in Figure 4C).

As another approach to target S1P1, we used small interfering RNA to reduce expression of S1P1 in macrophages. We achieved a 40% knockdown of S1P1 in RAW macrophages. This resulted in an \approx 40% reduction in the ability of either S1P or SEW2871 to either induce Arg I or suppress iNOS or TNF α mRNA expression (see Figure I in the online data supplement). The S1P1 small interfering RNA reduced S1P action in S1P2KO macrophages (supplemental Figure I), again supporting the notion that the S1P1 receptor is mediating many of the antiinflammatory effects of S1P on macrophage function.

iNOS Promoter Activity in RAW264.7 Macrophages

Because iNOS is such an important mediator of macrophage inflammatory responses, we wanted to further examine the regulation of iNOS promoter activity by S1P. We cloned a 1.7-kb region of 5'-flanking DNA located upstream of the transcriptional start site of the murine iNOS gene. This promoter region contains putative NF κ B, activator protein (AP)-1, and STAT-1 response elements (Figure 5A). Xie et al have shown that the LPS-mediated induction of iNOS transcriptional activity is primarily attributable to 1 of 2 NF κ B binding sites (termed NF κ B2 in Figure 5A) located within this promoter region.²⁵ We subcloned this 1.7-kb iNOS promoter region into pGL3-luciferase and transfected RAW macrophages. We found that LPS stimulated iNOS promoter activity by 400%, whereas S1P significantly reduced the LPS-mediated activation of the iNOS promoter (Figure 5B). Next, we mutated the distal NF κ B site that confers LPS induction of iNOS (termed NF κ B2 in Figure 5C). Mutation of this single NF κ B site reduced the ability of LPS to induce iNOS promoter activity by 75% to 80% (Figure 5C). However, although the bulk of the S1P effect was lost, there remained slight residual S1P activity (\approx 25%) in the absence of this functional NF κ B2 site (Figure 5C).

Because we previously reported that S1P inhibits NF κ B translocation to the nucleus in aortic endothelial cells,²⁶ we focused our efforts on NF κ B-mediated regulation of iNOS and TNF α . Using a NF κ B promoter-reporter construct that contained 3 tandem NF κ B elements linked to the luciferase gene, we confirmed that LPS activated NF κ B and that S1P significantly reduced NF κ B activation (Figure 6A). NF κ B resides in the cytosol in an inactive state, where it is sequestered by I κ B. Degradation of I κ B frees NF κ B, allowing it to mobilize to the nucleus to initiate gene transcription. We found that S1P both reduced the amount of p65-NF κ B that mobilized to the nucleus and also increased the amount of I κ B remaining in the cytosol (Figure 6B and 6C). Thus, we anticipate that regulation of I κ B expression is 1 mechanism by which S1P reduces NF κ B activation to regulate expression of inflammatory genes, such as iNOS and TNF α , in macrophages.

Finally, we confirmed that S1P acts through the NF κ B pathway to regulate TNF α secretion. As shown in supplemental Figure II, macrophages incubated with LPS show significant elevations in TNF α secretion. Treatment of macrophages with a well-characterized NF κ B inhibitor caused a dose-dependent reduction in TNF α secretion by these macrophages. S1P was unable to further reduce TNF α secretion, again supporting the notion that S1P inhibits NF κ B signaling in macrophages.

Discussion

Atherosclerosis development in the aortic wall is initiated by endothelial and monocyte/macrophage activation. The present study demonstrates a role for S1P in mediating antiinflammatory actions in macrophages by switching the macrophage phenotype from a proinflammatory classic phenotype to an antiinflammatory alternative phenotype primarily through action on the macrophage S1P1 receptor. LPS stimulates macrophage production of proinflammatory cytokines such as TNF α . In the present study, we show that S1P significantly reduces LPS-mediated expression of proinflammatory cytokines and the enzyme iNOS and stimulates Arg I expression in macrophages, indicating that S1P promotes an antiinflammatory macrophage phenotype (see Figure 2). This notion of phenotypic switching in macrophages by S1P was further supported by studies showing a direct effect of S1P on macrophage Arg and iNOS enzymatic activities (Figure 3). In addition to upregulation of Arg I, we also found increased production of the antiinflammatory cytokines transforming growth factor- β and IL-10 in S1P-treated macrophages (data not shown). Thus, S1P promotes a novel macrophage phenotype that is antiinflammatory in nature.

Furthermore, in the present study, we observed antiinflammatory effects of S1P at concentrations of 100 nmol/L to 1 μ mol/L, although the maximal effect on induction of Arg I and reduction of iNOS was at 500 nmol/L S1P rather than 1 μ mol/L S1P. There is an inverse relationship between the concentration of S1P and its effectiveness in modulating LPS responses in macrophages (Figure 2A). We have found this to be the case in studies of endothelial cells.²⁷ The physiological concentrations of S1P in blood are in the nanomolar range; the affinity of S1P for the S1P class of receptors lies in the nanomolar range.⁹ Consistently, we have found that 1 to 10 μ mol/L concentrations of S1P actually blunt the antiinflammatory effects of S1P in both macrophages and endothelial cells in a dose-dependent manner. One likely explanation for such findings is that the higher concentrations of S1P may activate receptors (other than S1P receptors) in a nonspecific manner that results in stimulation of proinflammatory signaling pathways.

Using a combination of KO mice and pharmacological reagents, we found that most of the phenotypic switching is driven by S1P action on the S1P1 receptor. S1P1-deficient mice display embryonic lethality, so we used SEW2871, a pharmacological agonist of the S1P1 receptor, and VPC44116, a pharmacological antagonist of the S1P1 receptor, to delineate the role of S1P1 in mediating these effects in macrophages. Because macrophages express only S1P1 and S1P2 receptors (Figure 1), we used S1P2-deficient mice to delineate the role of S1P2 in these processes. We found that S1P1 receptor signaling was responsible for the upregulation of Arg I and the downregulation of TNF α and iNOS (Figures 2 through 4 and supplemental Figure I). We confirmed that iNOS, TNF α , and Arg I regulation was mediated by the S1P1 receptor in that S1P2-deficient macrophages responded in a similar manner as wild-type macrophages to S1P and SEW2871 (Figure 3). We did observe that the response of S1P2-deficient macrophages to LPS, S1P, and SEW2871 was blunted compared with wild-type macrophages. It is possible that S1P could activate S1P2 to somehow oppose signaling through S1P1 and, thereby, reduce the antiinflammatory effect of the macrophage S1P-S1P1 axis. The role of S1P2 in macrophage phenotypic switching is unknown; we anticipate that direct S1P signaling through S1P2 may also have antiinflammatory effects. Indeed, we did find that S1P2 mediated the upregulation of IL-10 in macrophages (data not shown). S1P caused a significant 4-fold induction of IL-10 mRNA and protein in macrophages from C57BL/6J mice, but this induction was not observed in macrophages isolated from S1P2-deficient mice and treated with S1P (data not shown). Thus, the S1P2 receptor does appear to play a role in regulating the phenotype of macrophages, and this role may also have significance in atherosclerosis through upregulation of Th2-like responses via IL-10 signaling.

Previously, we demonstrated that S1P decreases NF κ B nuclear translocation in endothelial cells.²⁶ Based on this knowledge, we wondered whether the S1P-mediated reduction of iNOS expression was through inhibition of NF κ B. As shown in Figure 5, we confirmed that NF κ B is an important transcription factor that regulates iNOS activation by LPS. Mutation of the NF κ B2 binding site in the murine iNOS promoter dramatically inhibited LPS-mediated activation of iNOS (Figure 5C). However, despite the mutation of the NF κ B2 site, S1P was still able to downregulate iNOS promoter activity by approximately 25%. In preliminary studies, we have found that S1P reduces AP-1 and STAT-1 activation (data not shown), so the additional action(s) of S1P on iNOS regulation could be either through inhibition of the remaining NF κ B1 site or through inhibition of AP-1 and/or STAT-1 activation. All 3 of these transcription factors have been shown to be important for iNOS activation by LPS, although NF κ B is believed to be the primary regulatory transcription factor. It is unlikely that S1P would have a direct effect on transcription factors or bind directly to DNA. Most likely, S1P inhibits an upstream target of the transcription factor. For instance, we found that S1P activates mitogen-activated protein kinase phosphatases (data not shown), which inhibit mobilization of c-Jun and extracellular signal-regulated kinase 1/2 to the nucleus for initiation of gene transcription. Inhibition of c-Jun translocation to the nucleus would reduce AP-1 activation,

for example. Furthermore, we found that S1P significantly reduces NF κ B activation in macrophages, and this appears to be attributable, at least in part, to regulation of I κ B expression (Figure 6). We have previously reported that S1P induces I κ B synthesis within 30 minutes of incubation, which is very much within the time-frame of the present studies.²⁶ Taken together, our data indicate that the primary regulation of iNOS by LPS is through NF κ B and that S1P serves to reduce NF κ B activation, at least in part, through regulation of I κ B expression. Our data also suggest that regulating NF κ B activation may serve as an important signaling component for macrophage phenotypic switching in the vascular wall.

In summary, we show that S1P promotes phenotypic switching of macrophages to a novel, alternative antiinflammatory phenotype. These macrophages show decreased NF κ B activation and have reduced production of proinflammatory cytokines and nitric oxide. This novel antiinflammatory macrophage phenotype is mostly driven by the S1PS1P1 receptor axis. Therefore, S1P could provide a beneficial therapeutic effect for reducing macrophage-mediated inflammation in the vessel wall through specific activation of the S1P1 receptor.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Macrophages express S1P1 and S1P2 receptors. Expression of S1P receptor mRNA in C57BL/6J and S1P2-deficient bone marrow-derived macrophages were analyzed by conventional RT-PCR. There was no detectable expression of S1P3, S1P4, or S1P5 mRNAs. There was no compensatory upregulation of S1P1 in the S1P2-deficient macrophages. β -Actin was used as a control for sample loading. Data represent pooled RNA samples from 3 mice per group.

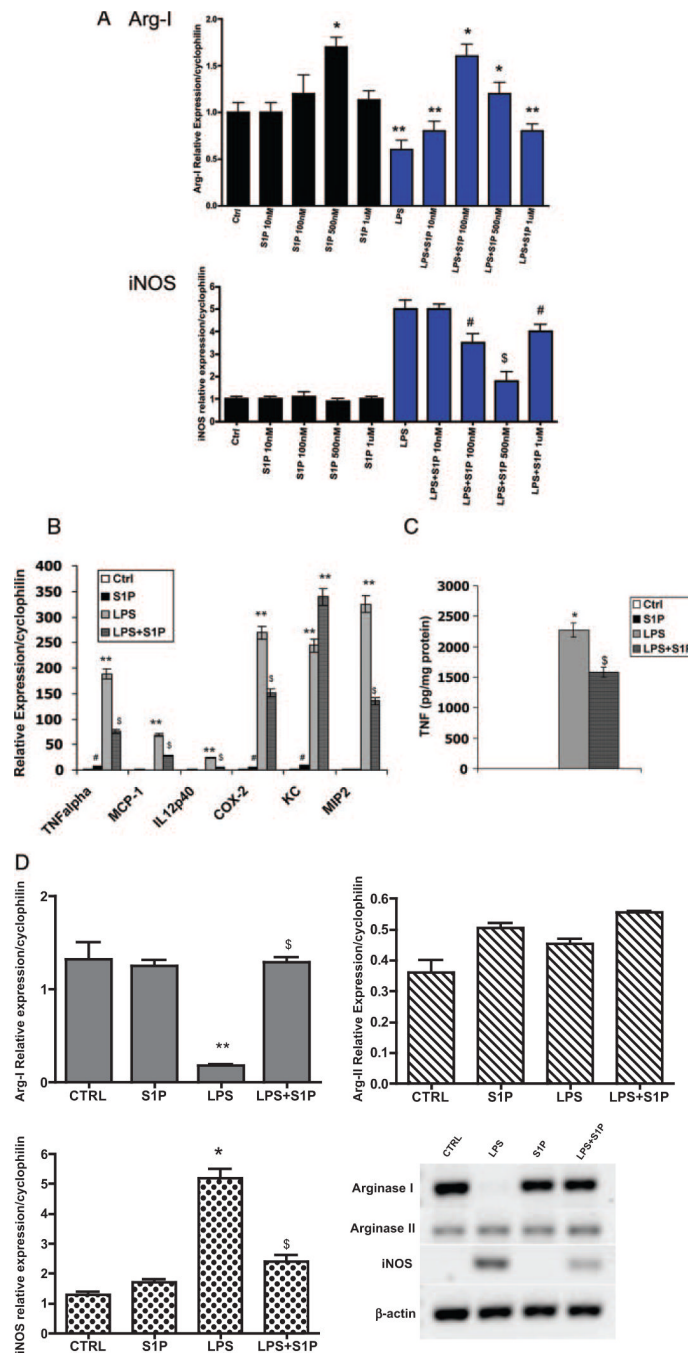


Figure 2.

S1P reduces LPS-induced inflammatory gene expression. A, S1P dose response. Total RNA was isolated from C57BL/6J peritoneal macrophages treated with S1P at concentrations of 10 nmol/L, 100 nmol/L, 500 nmol/L, and 1 µmol/L in the absence (black bars) or presence (blue bars) of 10 ng/mL LPS for 4 hours. Quantitative real-time PCR for murine Arg I and iNOS were performed. Cyclophilin was measured as a housekeeping gene for normalization purposes. Data are expressed using the relative expression method using B6 control as set to 1. * $P < 0.001$ vs control (Ctrl), ** $P < 0.0001$ vs Ctrl, # $P < 0.01$ vs LPS, \$ $P < 0.001$ vs LPS by ANOVA. Data are from 3 independent experiments using 4 mice per group. B, Inflammatory gene expression. Cytokine mRNA expression was determined using quantitative real-time

PCR. C57BL/6J peritoneal macrophages were treated with media alone (Ctrl) or with 10 ng/mL LPS in the absence or presence of 500 nmol/L S1P for 4 hours. $**P < 0.0001$ vs Ctrl, $\$P < 0.005$ vs LPS, $\#P < 0.05$ vs Ctrl by ANOVA. C, TNF α secretion. TNF α secretion by C57BL/6J peritoneal macrophages was measured using ELISA. Values were normalized to total cellular protein. $*P < 0.001$ vs Ctrl, $\$P < 0.005$ vs LPS by ANOVA. D, Arg I and iNOS expression. Levels of mRNA expression in C57BL/6J peritoneal macrophages were determined using quantitative real-time PCR as described in Materials and Methods and are graphically shown. $**P < 0.0001$ vs Ctrl, $\$P < 0.009$ vs LPS, $*P < 0.001$ vs Ctrl by ANOVA. Data represent 5 mice per group performed in triplicate. Conventional RT-PCR products of Arg I, Arg-2, iNOS, and β -actin are also shown in a representative gel.

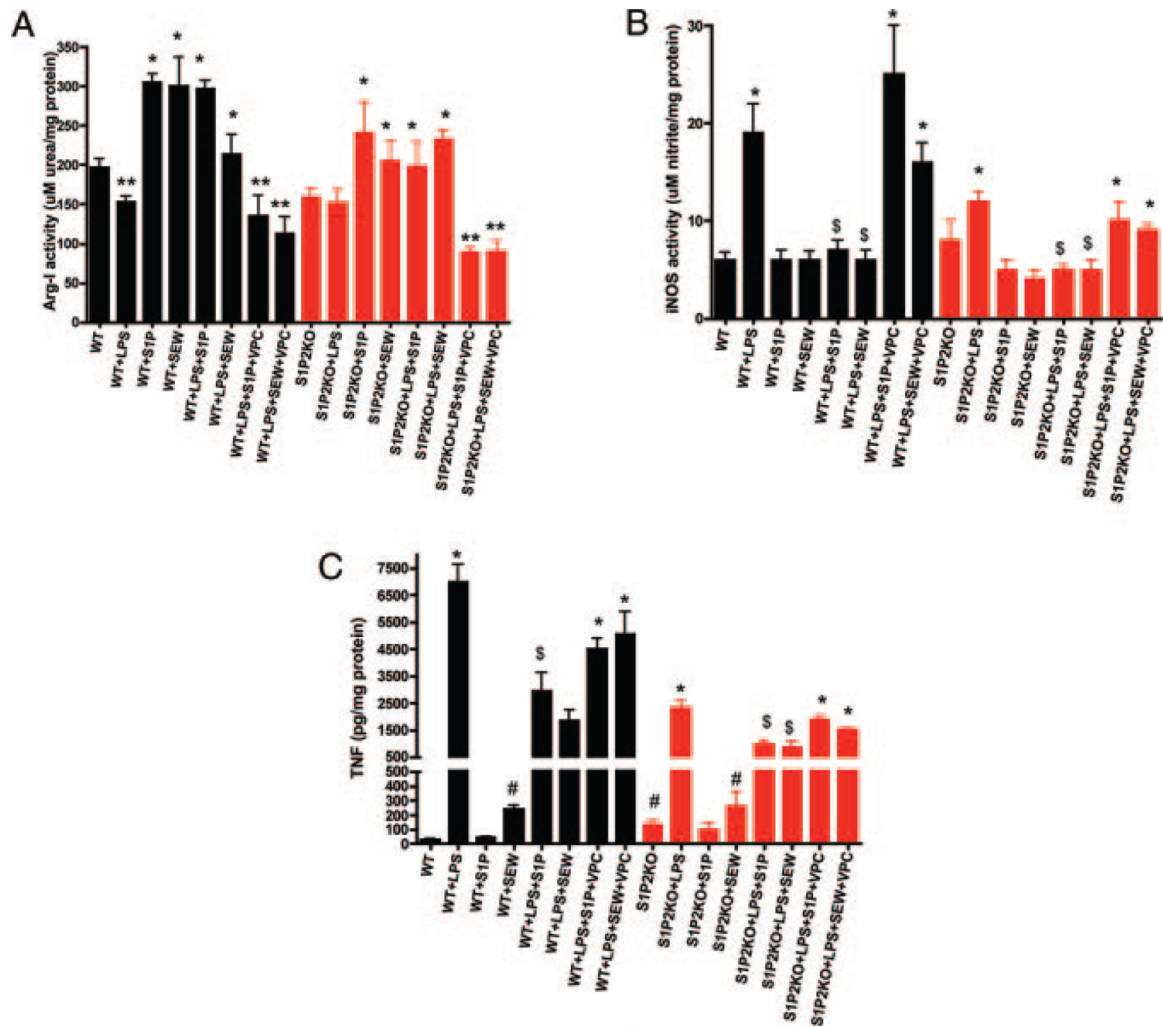


Figure 3.

The S1P1 receptor is antiinflammatory in macrophages. Bone marrow–derived macrophages from either wild-type littermate controls (WT) (black bars) or S1P2-deficient (S1P2KO; red bars) mice ($n=5$ mice per group) were treated with LPS (10 ng/mL) for 4 hours. In some cases, macrophages were treated with 500 nmol/L S1P, 1 μ mol/L SEW2871 (+SEW), or with 10 μ mol/L VPC44116 (+VPC) for 4 hours. A, Arg activity. Arg activity was quantified in cell lysates by measuring the conversion of L-arginine to urea. * $P<0.001$ vs WT, ** $P<0.01$ vs WT by ANOVA. B, iNOS activity. Total nitrate and nitrite levels were measured with the Griess reagent as described in Materials and Methods. * $P<0.001$ vs WT, \$ $P<0.01$ vs LPS by ANOVA. C, TNF α secretion. TNF α levels were measured using ELISA. * $P<0.001$ vs WT, # $P<0.05$ vs WT, \$ $P<0.01$ vs LPS by ANOVA.

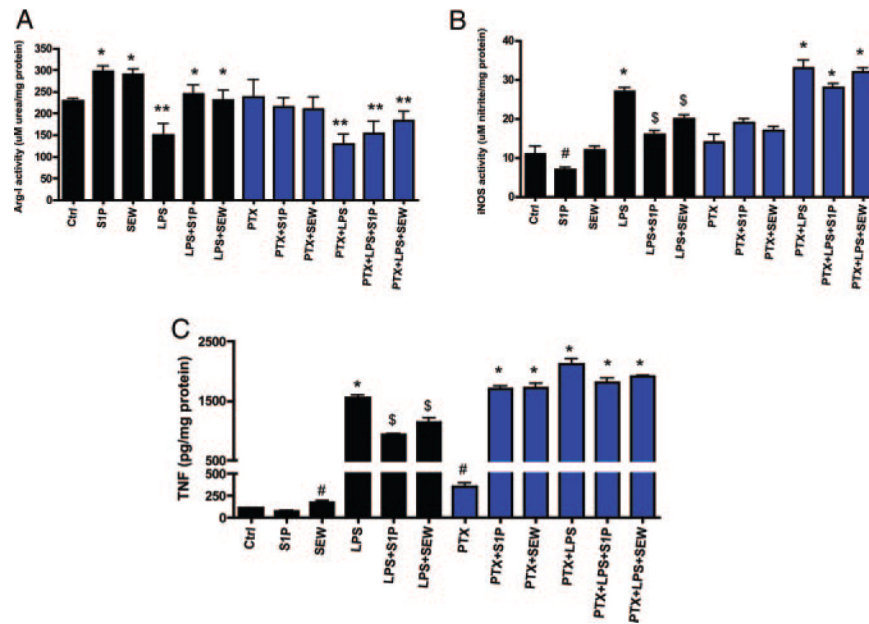


Figure 4.

Uncoupling of $G\alpha i$ signaling in macrophages blocks S1P action. Peritoneal macrophages from C57BL/6J mice (black bars) were incubated with media alone (Ctrl) or with 10 ng/mL LPS in the absence or presence of 500 nmol/L S1P for 4 hours. In some cases, cells were pretreated overnight with pertussis toxin (PTX) (blue bars) before LPS or S1P incubation to uncouple $G\alpha i$ signaling. A, Arg activity. Arg activity was quantified in cell lysates by measuring the conversion of L-arginine to urea. * $P < 0.001$ vs Ctrl, ** $P < 0.01$ vs Ctrl by ANOVA. B, iNOS activity. Total nitrate and nitrite levels were measured with the Griess reagent as described in Materials and Methods. # $P < 0.05$ vs Ctrl, * $P < 0.001$ vs Ctrl, \$ $P < 0.01$ vs LPS by ANOVA. C, TNF α secretion. TNF α levels were measured using ELISA. # $P < 0.05$ vs Ctrl, * $P < 0.001$ vs Ctrl, \$ $P < 0.01$ vs LPS by ANOVA.

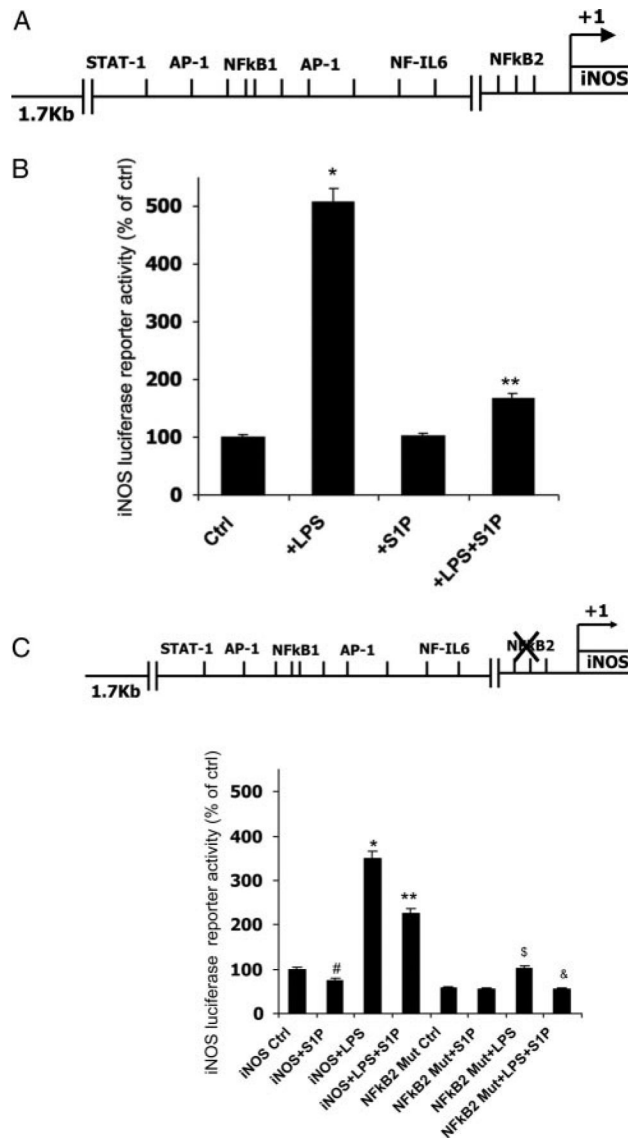


Figure 5. S1P modulates mouse iNOS promoter activity through the transcription factor NFκB. A, Diagram of putative transcription factor binding sites in the 1.7-kb murine iNOS promoter region. B, iNOS promoter-reporter activity. RAW264.7 macrophages were transiently transfected with a luciferase reporter plasmid containing 1.7 kb upstream of the transcriptional start site of the murine iNOS promoter. At 24 hours after transfection, macrophages were incubated in media alone (Ctrl), 10 ng/mL LPS (+LPS), 500 nmol/L S1P (+S1P), or both (+LPS +S1P) for 24 hours. Cells were harvested, and luciferase activity was measured. Luciferase values were normalized to *Renilla* luciferase. * $P < 0.001$ vs Ctrl, ** $P < 0.001$ vs LPS by ANOVA. Values are set to 100% for comparison between transfections; $n = 4$ experiments performed in triplicate. C, Mutant iNOS promoter-reporter activity. RAW264.7 macrophages were transfected with a luciferase reporter plasmid containing the 1.7 kb upstream of the transcriptional start site of the murine iNOS promoter in which the NFκB site no. 2 was mutated (NFκB2 Mut). Luciferase values were normalized to *Renilla* luciferase values. Values are set to 100% for comparison between transfections; $n = 3$ experiments performed in triplicate.

* $P < 0.001$ vs Ctrl, ** $P < 0.005$ vs LPS, # $P < 0.05$ vs Ctrl, \$ $P < 0.01$ vs NF κ B Mut Ctrl, & $P < 0.02$ vs NF κ B Mut+LPS by ANOVA.

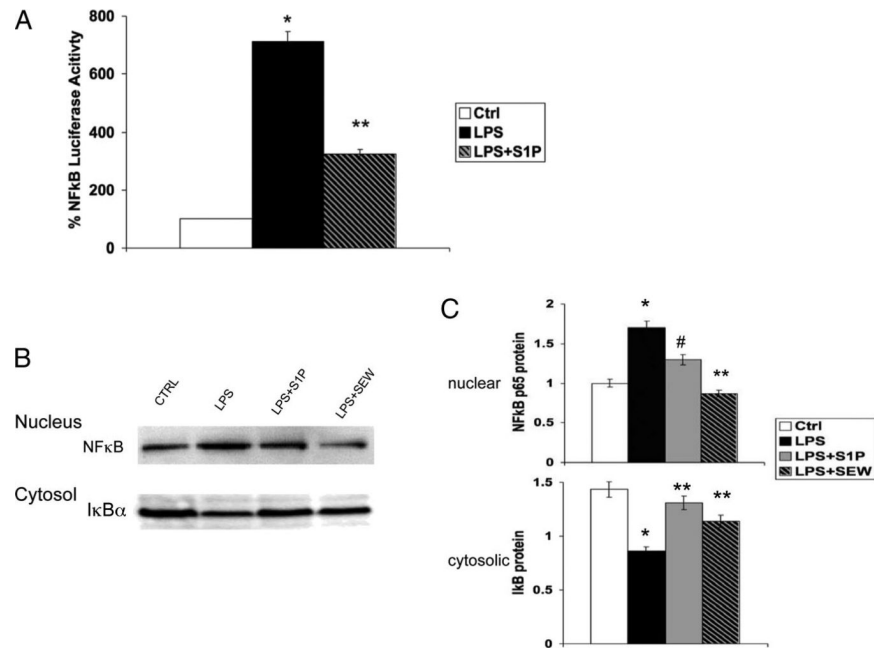


Figure 6.

S1P modulates NFκB activation in macrophages. A, NFκB reporter activation. RAW264.7 macrophages were transfected with a NFκB-luciferase reporter plasmid. After 48 hours, cells were incubated in media alone (Ctrl), 10 ng/mL LPS (+LPS), or LPS +500 nmol/L S1P (+LPS+S1P) for 4 hours. Luciferase activity was then measured. * $P < 0.001$ vs Ctrl, ** $P < 0.001$ vs LPS by ANOVA. B, Bone marrow-derived macrophages from C57BL/6J mice were incubated with 10 ng/mL LPS, 500 nmol/L S1P, or 1 μmol/L SEW2871 for 2 hours. Nuclear and cytosol extracts were collected and analyzed by SDS-PAGE for NFκB p65 and IκBα. A representative immunoblot is shown. Top, Nuclear extract probed with anti-NFκB p65 antibody. Bottom, Cytosolic extract probed with anti-IκBα antibody. C, Densitometry. Graphical representation of immunoblots in B normalized to histone H1 (nuclear) and tubulin (cytosol) from 3 independent experiments. * $P < 0.001$ vs Ctrl, # $P < 0.003$ vs LPS, ** $P < 0.001$ vs LPS by ANOVA.