**Sphingosine-1-Phosphate Receptor 3 Promotes Recruitment of Monocyte/Macrophages in Inflammation and Atherosclerosis**

Petra Keul,* Susann Lucke,* Karin von Wnuck Lipinski, Constantin Bode, Markus Gräler, Gerd Heusch, Bodo Levkau

**Rationale:** The role of sphingosine-1-phosphate (S1P) and its receptors in the pathogenesis of atherosclerosis has not been investigated.

**Objective:** We hypothesized that the S1P receptor 3 (SIP₃) plays a causal role in the pathogenesis of atherosclerosis.

**Methods and Results:** We examined atherosclerotic lesion development in mice deficient for SIP₃ and apolipoprotein (Apo)E. Although SIP₃ deficiency did not affect lesion size after 25 or 45 weeks of normal chow diet, it resulted in a dramatic reduction of the monocyte/macrophage content in lesions of SIP₃⁻/⁻/ApoE⁻/⁻ double knockout mice. To search for putative defects in monocyte/macrophage recruitment, we examined macrophage-driven inflammation during thioglycollate-induced peritonitis. Elicited peritoneal macrophages were reduced in SIP₃-deficient mice and expressed lower levels of tumor necrosis factor-α and monocyte chemoattractant protein-1. Bone marrow–derived SIP₃-deficient macrophages produced less MCP-1 in response to lipopolysaccharide stimulation. In vitro, S1P was chemotactic for wild-type but not SIP₃-deficient peritoneal macrophages. In vivo, SIP concentration increased rapidly in the peritoneal cavity after initiation of peritonitis. Treatment with the SIP analog FTY720 attenuated macrophage recruitment to the peritoneum. Studies in bone marrow chimeras showed that SIP₃ in both hematopoietic and nonhematopoietic cells contributed to monocyte/macrophage accumulation in atherosclerotic lesions. Finally, SIP deficiency increased the smooth muscle cell content of atherosclerotic lesions and enhanced neointima formation after carotid ligation arguing for an antiproliferative/antimigratory role of SIP₃ in the arterial injury response.

**Conclusions:** Our data suggest that SIP₃ mediates the chemotactic effect of S1P in macrophages in vitro and in vivo and plays a causal role in atherosclerosis by promoting inflammatory monocyte/macrophage recruitment and altering smooth muscle cell behavior. *(Circ Res. 2011;108:314-323.)*

**Key Words:** sphingosine-1-phosphate (S1P) ■ atherosclerosis ■ macrophages ■ inflammation ■ vascular biology ■ lipid metabolites

Sphingosine-1-phosphate (S1P) is a bioactive lipid with key functions in the immune, inflammatory, and cardiovascular systems. S1P is abundant in plasma (200 to 1000 nmol/L⁻¹), where it is contained mainly in the high-density lipoprotein fraction in a biologically active form. Accordingly, plasma S1P levels correlate positively with plasma high-density lipoprotein cholesterol and apolipoprotein AI. As a component of both plasma and interstitial fluid, S1P interacts with all vascular and nonvascular cell types that participate in the pathogenesis of atherosclerosis. All these cells have functional SIP receptors and respond to SIP in a number of different ways, which has prompted the question whether SIP and its receptors may play a role in the pathogenesis of atherosclerosis. A positive association between serum S1P levels and the severity of coronary artery stenosis has been described in humans. The effect of the SIP analog FTY720 on mouse atherosclerosis has been elucidated in 3 independent studies including one from our group. Two of these studies have shown FTY720 to attenuate the development of atherosclerosis in apolipoprotein E-deficient (ApoE⁻/⁻) and LDL-receptor deficient (LDL-R⁻/⁻) mice, respectively, whereas the third observed no effect on plaque size. Mechanically, FTY720 does not allow discriminating between the roles of individual SIP receptors as it engages 4 of 5 SIP receptors and exhibits different receptor desensitization kinetics from SIP. The mechanism of how FTY720 inhibits atherosclerosis has also remained unknown in view of the variety of its...
potentially atheroprotective effects: it stimulates nitric oxide production in endothelial cells, inhibits the production of reactive oxygen species and chemokines such as monocyte chemoattractant protein (MCP)-1, alters the production of inflammatory cytokines, and acts as a potent immunosuppressant. As the S1P receptor 3 (S1P3) appears to contribute to several of these effects, we directly addressed its role in atherosclerosis and inflammation in the present study. For this, we have examined atherosclerotic lesion development in S1P3−/− ApoE−/− double knockout mice as well as in bone marrow chimeras, and studied the recruitment of inflammatory monocyte/macrophages during thioglycollate-induced sterile peritonitis in S1P3−/− mice. Our study provides in vitro and in vivo evidence that the S1P3 receptor on monocyte/macrophages affects their migration properties and inflammatory potential and promotes their recruitment to inflammation sites and atherosclerotic lesions.

Methods

An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

Mice

S1P3−/− mice were crossbred to the C57BL/6 background for more than 10 generations and subsequently to ApoE−/−. FTY720 was administered orally at 1.25 mg/kg per day.

Hematology and Flow Cytometry

Total and differential white blood cell counts were measured in EDTA-blood from the retroorbital plexus using an automated Animal ABC Coulter Counter. Flow cytometry of blood, bone marrow and peritoneal exudate was performed with fluorochrome-conjugated antibodies to CD4, CD8, CD45, B220, Gr-1, I-Ab, Ly-6G, F4/80, CD11b, and CD115 on a FC500 flow cytometer.

Immunohistochemistry

Immunohistochemistry was performed on adjacent sections with antibodies to αSMA, Mac-2 and sphingosine kinase (Sphk1) (a kind gift from Dr Lina Obeid, Medical University of South Carolina). Collagen content was assessed by picrosirius red staining and polarized light. Immunostaining for every marker was performed in a single procedure. Automated color segmentation of images was performed, and a color threshold of chroomagen stain defined using a negative isotype-matched control antibodies. The area containing all positive pixels was expressed as percentage of total plaque area.

Flow Cytometry of Single Aortae

Single-cell suspensions from aortae were prepared following a protocol graciously provided by Prof Klaus Ley (Division of Inflammation Biology, La Jolla Institute for Allergy & Immunology), as described with minor modifications. Briefly, mice were perfused with perfusion saline and the carefully dissected aorta cut into small pieces and digested in an enzyme cocktail. Cells were stained with CD45-PE-Cy7, CD11b-APC (clone M1/70 or Mac-1) and I-Ab-PE as described. Annexin-V–FITC served to differentiate living from apoptotic cells. Cells negative for Annexin-V were back-gated to CD45− and determined as percent from total aortic surface area using the Cavalieri stereologic method (Σ[lesion area]×(distance; 75 μm)]). Lesion area and vessel area in the aortic root were calculated from 4 consecutive 0.05 μm sections of 40 μm apart over the entire root. The volume fraction of the lesion was calculated as lesion volume (Σ[lesion area including valve area]×(distance; 40 μm)) divided by vessel volume (Σ[vessel area]×(distance; 40 μm)).

Carotid Ligation, Smooth Muscle Cell Proliferation, and Migration

The left carotid artery was ligated at the bifurcation as previously described and serially sectioned over its entire length 4 weeks after surgery. Every 10th section starting at the ligation site was stained with H&E and neointimal and medial areas and neointimal cells were quantified by histomorphometry. Smooth muscle cells (SMCs) were isolated from aortae (6 to 7 aortae per preparation) by enzymatic digestion as described, plated on Vitrogen100, and used for experiments up to the sixth passage. For proliferation studies, SMCs were serum-starved for 24 hours and cultured in 1% FCS in the presence or absence of 1 μmol/L S1P and 1 nmol/L platelet-derived growth factor (PDGF), respectively, added every other day for 4 days.
ApoE

aorta (determined en face using oil red O) nor lesion volume
and 45 weeks, respectively. Neither lipid accumulation in the
membrane in one field of view at 10x magnification as described.18 Five
after 4 hours by counting the cells migrated to the lower side of the
contrast, the monocyte/macrophage content and the SMC con-

Lesion Volume but Dramatically Reduces
Macrophage Content in Atherosclerotic
Lesions of S1P3−/−/ApoE−/− Mice
To analyze the effect of S1P3 deficiency on atherosclerotic
lesion formation, we backcrossed S1P3 knockout mice to the
C57BL/6 background for 10 generations and crossbred them
with ApoE−/− deficient mice. We then compared size and
composition of atherosclerotic lesions in the aorta and the
brachiocephalic artery (BCA) after 45 weeks of normal chow diet using
the Cavalieri stereologic method. *P<0.05; n.s. indicates not significant.

Statistical Analysis
Each experiment was performed at least 3 times as indicated, and data
presented as means±SEM. Statistical significance was evaluated by a
nonparametric Wilcoxon–Mann–Whitney test. Two-sided probability
values of less than 0.05 were considered significant.

Results
S1P3 Deficiency Has No Effect on Atherosclerotic
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composition of atherosclerotic lesions in the aorta and the
brachiocephalic artery (BCA), respectively, of S1P3−/−/ApoE−/− and
S1P3−/−/ApoE−/− mice fed a standard chow diet for 25
and 45 weeks, respectively. Neither lipid accumulation in the
aorta (determined en face using oil red O) nor lesion volume
(determined by the Cavalieri method in the BCA) was different
between S1P3−/−/ApoE−/− and ApoE−/− mice (Figure 1). In
contrast, the monocyte/macrophage content and the SMC con-
reduced macrophage content of atherosclerotic lesions of S1P3−/−/ApoE−/− mice. To test this, we used the thioglycollate-induced peritonitis model as a classical model to study macrophage-driven sterile inflammation (Figure 4A). In this model, we observed a dramatic reduction in the peritoneal numbers of F4/80+Gr-1+ macrophages in S1P3-deficient mice compared to controls 48 and 96 hours, respectively, after initiation of peritonitis (Figure 5A and 5B; Online Figure II). In contrast, the influx of neutrophils measured at day 1 was not different between the 2 genotypes (9.15 ± 2.99 versus 7.45 ± 1.84 × 106/mouse).

We then compared the expression of the S1P1, S1P2, and S1P3 receptors both in thioglycollate-elicited peritoneal macrophages and BMDMs by real-time PCR (Figure 4B and 4C). In both macrophage types, expression of S1P1, and S1P2 was 2 to 3-fold higher than that of S1P3 (determined as absolute copies of gene transcript per 1000 copies GAPDH; Figure 4B). Gene transcript number was comparable between peritoneal and BMDMs for all 3 S1P receptors (Figure 4C).

S1P3-Deficient Macrophages Exhibit an Altered Cytokine Expression Profile Compared With Controls

We then tested for alterations in the expression of inflammatory chemokines, cytokines, and cytokine receptors in S1P3−/− peritoneal macrophages by real-time PCR. We found MCP-1 and tumor necrosis factor (TNF)α gene expression to be reduced by 70% to 80% in S1P3−/− macrophages (Figure 5C), whereas other genes were unchanged at the mRNA (interleukin [IL]-4, IL-6, IL-10, IL-1α, MIP-1α, GM-CSF, CCR2; Figure 5C) or protein level (iNOS, arginase-1, CCR2, CXCR7, CX3CR1, and CD80; data not shown). In agreement, protein levels of MCP-1 were reduced more than 150-fold in the peritoneal exudates of S1P3−/− mice compared to controls as measured by ELISA (Figure 5C, inset). Incubation of BMDMs with S1P did not have any effect on basal or lipopolysaccharide (LPS)-stimulated MCP-1 expression in wild-type or S1P3−/−/ApoE−/− mice (Figure 5D, inset).

SIP Levels Increase in the Inflamed Peritoneum In Vivo; SIP Chemoattracts Macrophages via S1P3 In Vitro; FTY720 Abolishes Peritoneal Macrophage Recruitment In Vivo

We measured S1P levels in the peritoneal fluid with a maximum at 24 hours and a subsequent decrease (Figure 6A). We hypothesized that such an S1P increase might be biologically important and tested it in 2 ways. Firstly, we examined whether S1P may be a chemoattractant for thioglycollate-elicited peritoneal macrophages in vitro. We found MCP-1 and tumor necrosis factor (TNF)α gene expression to be reduced by...
wild-type macrophages migrated toward a 1 μmol/L S1P gradient in Boyden chamber experiments (Figure 6B). In contrast, S1P3−/− macrophages did not migrate toward S1P (Figure 6B), suggesting that S1P3 is responsible for mediating the chemotactic response to S1P. Secondly, we eliminated any biological effects that S1P gradients may have in vivo by treating mice with the S1P analog FTY720 as previously described for lymphocytes.20 This resulted in a ~80% reduction in the number of elicited macrophages after thioglycollate application (Figure 6C), suggesting that S1P gradients may be required for effective monocyte/macrophage recruitment to the inflamed peritoneum in vivo.

**Sphingosine Kinase 1 Is Expressed in Lesions of Atherosclerosis**

Immunohistochemistry for Sphk1 of atherosclerotic lesions of ApoE−/− mice showed that the enzyme was abundantly expressed there and colocalized mainly with macrophages (Figure 6D). This suggested that S1P-synthesizing enzymes such as Sphk1 may promote local production of S1P in atherosclerotic lesions.

**S1P3 in Both Hematopoietic and Nonhematopoietic Cells Is Required for Monocyte/Macrophage Accumulation in Atherosclerotic Lesions**

To examine the role of the monocyte/macrophage S1P3 in the recruitment of inflammatory cells to atherosclerotic lesions, we generated bone marrow chimeras by reconstituting the bone marrow of sublethally irradiated ApoE−/− mice with that of S1P3−/−/ApoE−/− mice and vice versa. Successful bone marrow substitution was verified by diagnostic PCR for the deleted and wild-type S1P3 allele on genomic DNA from total bone marrow chimeras (26.25% in the S1P3−/−/ApoE−/− bone marrow chimeras compared to the S1P3−/−/ApoE−/−/ApoE−/− mice, p = 0.002). Chimeras and ApoE−/− (donor)→ApoE−/− (recipient) chimeras were analyzed by flow cytometry. A, Monocytes were identified as F4/80+CD115+ cells (dot plots) and analyzed for expression of Ly-6C, Ly-6G, and F4/80. B, Neutrophils were identified using CD11b and Ly-6G, and analyzed for expression of Ly-6C. C, General hematologic parameters and cholesterol levels in plasma of ApoE−/− and S1P3−/−/ApoE−/− are shown as means ± SEM.

**Figure 3. No differences in myeloid cell populations and monocyte subsets in the peripheral blood of S1P3−/−/ApoE−/− and ApoE−/− mice.** Peripheral blood monocytes and neutrophils of 45-week-old mice were analyzed by flow cytometry. A, Monocytes were identified as F4/80+CD115+ cells (dot plots) and analyzed for expression of Ly-6C, Ly-6G, and F4/80. B, Neutrophils were identified using CD11b and Ly-6G. C, General hematologic parameters and cholesterol levels in plasma of ApoE−/− and S1P3−/−/ApoE−/− are shown as means ± SEM.

**Neointima Formation After Carotid Ligation Is Increased in S1P3−/−/− Mice**

To follow up on the finding of an elevated SMC content in atherosclerotic lesions of S1P3−/−/ApoE−/− mice, we used the carotid artery ligation model.21,22 There, we observed a larger neointima formation in S1P3−/−/− mice compared to ApoE−/−→ApoE−/− bone marrow chimeras compared to ApoE−/−→S1P3−/−/ApoE−/− chimeras (26.25 ± 2.03% versus 19.51 ± 1.59%; Figure 7B and 7C). This suggested that expression of S1P3 in monocyte/macrophages partially contributes to their accumulation in atherosclerotic lesions. Analysis of the SMC content of the same lesions showed it was increased in the S1P3−/−/ApoE−/−→ApoE−/− bone marrow chimeras compared to ApoE−/−→S1P3−/−/ApoE−/− (5.89 ± 0.93% versus 3.10 ± 0.26%; Figure 7D).
PDGF-BB-induced SMC migration as published was equally effective in wild-type and S1P3−/− SMCs (Figure 8C).

Proliferation in response to PDGF-BB was similar in SMCs from both genotypes and was unaffected by S1P (Figure 8D).

Discussion

Our study is the first to address the role of S1P3 in the pathogenesis of atherosclerosis in vivo. Our initial observation was that macrophage content was decreased in atherosclerotic lesions of S1P3−/−/ApoE−/− double knockout mice. Using bone marrow chimeras, we could partially attribute this to the lack of S1P3 in hematopoietic cells. A possible scenario how this may occur is that monocyte/macrophages are chemotactically attracted toward an increased S1P level inside the lesion, to which they respond via their S1P3 receptor. This resembles the action of the bone fide chemokines in atherosclerosis. Several of our observations support such a scenario: S1P was chemotactic for macrophages in vitro; migration to S1P was dependent on the macrophage S1P3 receptor in vitro, and S1P3-deficient mice recruited less monocyte/macrophages in the peritonitis model. During peritonitis, S1P levels increased locally in the inflamed peritoneum, thereby providing a

Figure 4. Quantification of S1P1, S1P2, and S1P3 gene expression in peritoneal and BMDMs. A, Schematic of thioglycollate-induced peritonitis. B, Quantitative assessment of S1P receptor expression was performed in thioglycollate-elicited peritoneal macrophages (4 days after injection) and BMDMs from control and S1P3−/− mice by real-time PCR. The absolute copy number of S1P1, S1P2, and S1P3 is expressed per 1000 copies of GAPDH. *P<0.05 (expression of S1P3 compared to S1P1 and S1P2).

Figure 5. Altered recruitment of macrophages to the inflamed peritoneal cavity of S1P3−/− mice. A, Peritoneal macrophages (F4/80+ Gr-1−) are reduced in S1P3−/− mice 4 days after thioglycollate injection. Data are expressed as mean of 6 different mice per group. **P<0.01. B, Kinetics of macrophage recruitment in S1P3−/− mice. Peritoneal macrophage number was determined over a time course of 4 days. Data are expressed as a mean of 6 mice per group. *P<0.05. C, Cytokine expression in thioglycollate-elicited peritoneal macrophages of S1P3−/− mice compared to controls. Total macrophage RNA was isolated from control (n=10) and S1P3−/− (n=8) mice 4 days after thioglycollate injection and cytokine gene expression was analyzed by real-time PCR. Data of relative amounts are normalized to GAPDH and calculated by the 2−ΔΔCT method. *P<0.05; **P<0.01. Inset, Protein levels of MCP-1 in the peritoneal exudate as determined by ELISA (n=3 per group, measured in triplicate; *P<0.05). D, BMDMs from control and S1P3−/− mice were stimulated with LPS (100 ng/mL), S1P (1 μmol/L), and both, respectively, for 4 hours. Total RNA was isolated and real-time PCR for MCP-1 was performed with GAPDH as endogenous reference. Data are expressed as fold expression compared with unstimulated controls. A representative experiment performed in triplicate out of 4 is shown. **P<0.01. Inset, Fold induction of MCP-1 expression in S1P3−/− macrophages compared to C57BL/6 macrophages (set to 100%; mean of 4 experiments). *P<0.05.
Sphk1, macrophages (Mac-2), and SMCs in atherosclerotic lesions of ApoE–/– mice. FTY720 has been shown to reduce the macrophage content of atherosclerotic lesions of ApoE–/– mice by overloading the system with excess FTY720. This occurred in a manner that resembles the inhibition of lymphocyte migration out of lymph nodes by FTY720. In respect to atherosclerosis, FTY720 has been shown to reduce the macrophage content of atherosclerotic lesions of ApoE–/– mice. Although we could not measure the S1P content of atherosclerotic lesions directly, we found an abundant expression of Sphk1, which was colocalized mainly with macrophages. It is known that Sphk1 expression can be induced in vitro by several cytokines such as TNFα and IL-1β that are also present and active in atherosclerotic lesions in vivo. Furthermore, Sphk1 expression is increased in endothelial cells in models of acute lung injury induced by LPS or thrombin. Thus activated macrophages as well as vascular cells such as the endothelium may be potential sources of S1P in the lesion (as well as in the inflamed peritoneum in analogy).

Our finding that S1P is chemotactic for thioglycollate-elicited peritoneal macrophages in vitro is in agreement with its chemotactic effect in RAW264.7 monocytes as well as in mouse BMDMs. However, there are also reports of S1P lacking a chemotactic effect in mouse BMDMs but inhibiting migration toward C5a and CXCL12. Such differences may be attributable to the different tissue sources of the used macrophages, as well as their different activation and differentiation states. For instance, the S1P responsiveness of the thioglycollate-elicited inflammatory macrophages (used immediately after isolation for experiments as in our study)
may very well differ from that of BMDMs generated from hematopoietic precursors by long-term culture with M-CSF.27 In addition, different S1P receptors have apparently different effects on monocyte/macrophage migration: S1P2-deficient mice have been shown to recruit more peritoneal macrophages in the same peritonitis model as used in our study, whereas BMDMs from S1P3−/− mice were unresponsive to the migration-inhibitory effect of S1P on C5a and CXCL12.27 This has lead to the suggestion that plasma S1P restrains macrophages from migrating into inflammatory sites via S1P2.27 Another option may be that S1P2 retains macrophages inside the lesion once they have immigrated (provided the S1P content of the lesion is, indeed, increased). Our data suggest that macrophages use their S1P1 receptor to immigrate to such S1P-containing lesions. A scenario in which S1P2 prevents and S1P3 promotes monocyte/macrophage recruitment is reminiscent of the functional antagonism of 2 other S1P receptors, S1P1 and S1P2, in endothelial cell migration.28 The S1P1 receptor does not appear to play a role at least in macrophage recruitment to the inflamed peritoneum as shown using S1P1 hematopoietic chimeras27 and supported by our own data in mice carrying a conditional deletion of S1P1 in myeloid cells (S1P1LysMCcre−/−, data not shown).

Despite the somewhat macrophage-centric view we have adopted here, it must be clearly established that nonhematopoietic components also contribute to the altered monocyte/macrophage recruitment in S1P3-deficient mice. The macrophage content of lesions in the hematopoietic chimeras lacking S1P3 was significantly reduced but this reduction was modest compared to that in the global S1P3 knockout without bone marrow transplantation. In addition, wild-type mice transplanted with S1P3−/− bone marrow and S1P3−/− mice transplanted with wild-type bone marrow, respectively, showed no difference in peritoneal monocyte/macrophage recruitment after thioglycollate injection (Online Figure III), suggesting that S1P3 in the hematopoietic and the nonhematopoietic components is equally important. Whether its lack in either one is also sufficient to alter recruitment cannot be deduced from the data as we have not excluded possible effect of irradiation and stromal repopulation on the peritoneal response.

The most probable nonhematopoietic component in question is the endothelium: it is constantly exposed to the plasma S1P pool and needs it for maintenance of regular barrier function and prevention of vascular leakage.29 In addition, S1P and/or S1P1 agonists have been shown to inhibit monocyte adhesion to TNFα-activated endothelium30,31 as well as the recruitment of neutrophils to posts ischemic inflammation.32 The exact mechanisms have not been elucidated as several adhesion molecules and chemokines are differently affected by S1P and respond differently to low versus high S1P concentrations.4 However, there is also contrary evidence in favor of a proinflammatory role for S1P in the endothelium: Sphk1 is stimulated by inflammatory cytokines in endothelial cells and the S1P it generates stimulates COX-2 activity and PGE2 release24,25; it also mediates TNF-α-stimulated adhesion molecule expression and promotes neutrophil adhesion (for a comprehensive review, see elsewhere).4 Finally, the same cytokines that use S1P for the propagation of inflammation can also be restrained by the same S1P as taking place during inflammatory lung injury, where the initial increase in vascular permeability by LPS or PAR-1 is restored by their induction of S1P production.24,25 Thus, S1P appears to play a role both in the propagation and limitation of vascular inflammation. Therefore, any net effect S1P may have on monocyte/macrophage recruitment in vivo must be the result of simultaneous signaling by several S1P receptors on endothelial and inflammatory cells.

Finally, S1P signaling via the S1P3 receptor not only affects macrophage migration but also their activation profile: Thioglycollate-elicted S1P3−/− peritoneal macrophages exhibited reduced TNFα and MCP-1 expression suggesting a proinflammatory role for S1P3. Previously, an antiinflamma-
tory role for S1P1 and a proinflammatory one for S1P2, respectively, have been suggested based on S1P effects observed in BMDMs stimulated with LPS/IFN-γ. Although we did not see any S1P effects on basal or LPS/IFN-γ-stimulated cytokine expression (neither in wild-type nor S1P1/−/− BMDMs), we did observe a blunted MCP-1 response to LPS in S1P3/−/− macrophages. Thus S1P receptor signaling may affect cytokine expression of macrophages even in the absence of exogenous S1P. This may not only influence monocyte/macrophage function but also indirectly, via MCP-1, their recruitment in vivo.

The elevated SMC content we have seen in lesions of S1P3/−/−/ApoE−/− mice and the enhanced neointima formation after carotid ligation suggest that S1P3 also affects SMC phenotype. Despite numerous studies on S1P receptors in SMCs in vitro, only 2 studies have examined their role in vivo. In these, administration of a S1P1/S1P3 antagonist in rats inhibited neointima formation after balloon injury of the carotid34 and neointima formation after carotid ligation was enhanced in S1P3/−/− mice.17 The potent inhibition of PDGF-BB-induced migration by S1P and its lack of effect on basal migration that we see in our study confirm previous in vitro reports.18,28,35 The absent effect of S1P on proliferation has also been described before e. g. for human SMCs, although an enhanced proliferation by S1P has also been described for rat SMCs, where an S1P1/S1P3 inhibitor prevented it but did not alter basal proliferation.34 Although our data exclude S1P3 as being involved in these processes in vitro, they clearly argue for its importance in vivo. Thus, the in vivo mechanism behind the aberrant SMC behavior in the absence of S1P3 will need to be addressed in future studies, which need to take into consideration the fact that SMCs from different vessels express different S1P receptors and regulate them differently after vascular injury.18,28,35 Nevertheless, our carotid ligation data point to an antiproliferative/antimitogenic role of S1P1 on SMCs in the arterial injury response that may explain the shift toward the more SMC-rich plaque composition in S1P1−/−/ApoE−/− mice.

In summary, further studies are needed to dissect the role of S1P receptors on vascular endothelial, smooth muscle and hematopoietic cells in the context of atherosclerosis. This would help to design novel pharmacological approaches to atherosclerosis based on S1P agonists and antagonists.

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Disclosures

None.

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**Materials and Methods**

**Mice**
C57BL/6 mice were obtained from the Jackson Laboratory. S1P\textsubscript{3}\textsuperscript{-/-} mice were kindly provided by J. Chun (The Scripps, La Jolla, CA, USA) and were crossbred to the C57BL/6 background for more than 10 generations. ApoE\textsuperscript{-/-} and S1P\textsubscript{3}\textsuperscript{-/-} mice were crossbred to generate S1P\textsubscript{3}\textsuperscript{-/-}/ApoE\textsuperscript{-/-} double knockout mice. Male mice were used in all experiments. FTY720 was administered with the drinking water at a dose of 1.25 mg/kg/d as described\textsuperscript{1}.

**Hematology and flow cytometry**
Blood was collected from the retroorbital plexus into EDTA tubes at the time of sacrifice. Total and differential blood counts were measured using an automated Animal ABC Coulter Counter (VetABC, scil animal care company, Viernheim, Germany). For analysis of leukocytes in blood, bone marrow and peritoneal lavage by flow cytometry, red blood cells were lysed using ACK buffer (0.15 M NH\textsubscript{4}Cl; 1 mM KHCO\textsubscript{3}; 0.1 mM Na\textsubscript{2}EDTA; pH 7.2-7.4) for 2 minutes at room temperature. The cells were stained with fluorochrome-conjugated combinations of the following monoclonal antibodies: CD4 (Acris, Herford, Germany), CD8, CD45, B220, Gr-1 (reacts with Ly-6G and Ly-6C; Ly-6C is only expressed by monocytes), I-A\textsuperscript{b} and Ly-6G (all from BD Biosciences, Heidelberg, Germany), F4/80 (AbD Serotec, Düsseldorf, Germany), CD11b (Miltenyi Biotec, Bergisch Gladbach, Germany) and CD115 (eBioscience, Frankfurt, Germany) and were analyzed using a FC500 flow cytometer (Beckman Coulter, Krefeld, Germany). The size of each cell population was determined as a product of the total white blood cell count and the percentage of the respective cell population. Lipid analysis was performed using commercially available assays (Biocon Diagnostik, Vöhl/Marienheide, Germany).
Isolation of peritoneal and bone marrow macrophages

Peritoneal macrophages were collected after intraperitoneal injection of aged, sterile 3% thioglycollate (Sigma Aldrich, Munich, Germany) at the indicated times. To isolate them and obtain peritoneal fluid, 4 ml of pre-warmed PBS was injected into the abdominal cavity and aspirated. After centrifugation for 5 minutes at 1200 rpm and 4°C, the supernatant was frozen for subsequent S1P analysis, while the cells were counted and directly analyzed using flow cytometry or used for RNA isolation for gene expression studies. Bone marrow-derived macrophages were generated by the following procedure: Bone marrow was isolated from tibiae and femora by snipping off the ends of the bones and flushing out the bone marrow into a dish; cells were transferred into a polypropylene tube by passing them through a 70 µm cell strainer; after centrifugation (5 min, 1300 rpm, RT), red blood cells were lysed using ACK buffer and the washed cells were plated and cultured for 10 days with 1.5 ng/ml macrophage colony-stimulating factor (M-CSF; R&D Systems, Wiesbaden, Germany). Stimulation with LPS (Sigma Aldrich, Munich, Germany) and S1P (Biomol, Hamburg, Germany) was performed as indicated in the results section and total RNA was isolated as described below.

RNA isolation and quantitative PCR analysis of gene expression

Total RNA was isolated from mouse peritoneal macrophages and bone marrow-derived macrophages using RNaseasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s protocol. cDNA was synthesized using the Revert Aid First Strand cDNA Synthesis Kit (Fermentas, St. Leon-Rot, Germany). Quantitative real-time PCR was performed using an iCycler and iQ SYBR Green Supermix (BioRad, Munich, Germany) and Quantitect primer assays (Qiagen, Hilden, Germany). Absolute quantification of gene expression was performed with a standard concentration curve for S1P₁, S1P₂, S1P₃ and GAPDH transcripts generated from macrophage RNA beforehand using defined concentrations of PCR products for each amplified gene. The linear equation of the standard curve
allows a determination of the logarithm of the copy number of any unknown sample. The absolute copy number of S1P<sub>1</sub>, S1P<sub>2</sub> and S1P<sub>3</sub> was expressed as copies per 1000 copies GAPDH [gene (normalized) = copy number of target/copy number of reference]. Where relative quantification of gene expression was calculated, expression of genes was normalized to an endogenous reference (GAPDH or β-actin as indicated) and calculated by the 2<sup>-ΔΔCT</sup> method.

**S1P measurements**

Determination of S1P was performed by HPLC according to established protocols<sup>2</sup>. Briefly, lipids were extracted from plasma by successive addition of 1 ml of methanol, 200 µl of 6 M HCl and twice 2 ml of chloroform. Chloroform phases were retrieved by centrifugation, and chloroform was removed by vacuum-drying in a speed-vac. Subsequently, samples were dissolved in 200 µl of dioxane, 200 µl of 70mM K<sub>2</sub>HPO<sub>4</sub> and 200 µl of 9-fluorenylmethyl chloroformate (FMOC-Cl) solution for derivatization of sphingolipids. For chromatographic detection of sphingolipids (Merck-Hitachi Elite LaChrom System, VWR), 10 µl of sample was injected by cut-injection method with an injection-pump delivery rate of 1.3 ml/min into an eluent containing methanol, 70 mM K<sub>2</sub>HPO<sub>4</sub>, and H<sub>2</sub>O. Columns used for the separation of sphingolipids by reversed phase HPLC were a 250×4.6 mm Kromasil 100-5 C18 column and a 17×4 mm Kromasil 100-5 C18 pre-column (CS Chromatographie Service, Langerwehe, Germany) set to 35°C. Detection was performed with a fluorescence detector (excitation 263 nm, emission 316 nm).

**Bone marrow transplantation**

Mice were irradiated with 11 Gy and transplanted with 5 x 10<sup>6</sup> donor bone marrow cells via retroorbital injection the following day. At the time of sacrifice (25 weeks), diagnostic PCR with primers for deleted S1P<sub>3</sub> and the wildtype S1P<sub>3</sub> gene was performed with DNA isolated
from blood leukocytes (Qiagen, Hilden, Germany) to confirm complete bone marrow substitution. Peripheral blood cell counts were measured by VetABC and individual cell populations quantified by flow cytometry as described above.

Quantification of lesion volume

Quantification of atherosclerotic lesion volume in the brachiocephalic artery (BCA) and the aortic root was performed as previously described\(^3\). After sacrifice, mice were perfusion-fixed with 4.5% neutral-buffered formaldehyde and the BCA was dissected from the bifurcation off the aortic arch to the branching point of the right subclavian and common carotid artery, and embedded in paraffin. The entire BCA was serially sectioned in 5 µm sections. Beginning from a random start site within the first 75 µm, every 15\(^{th}\) section was stained with haemalaune and eosine. Images were captured with a Zeiss Axio Cam, and lesion area was quantified using AxioVision 4.6 software (Carl Zeiss Vision GmbH, Jena, Germany). The volume of the BCA lesion was determined using the Cavalieri stereologic method [\(\Sigma\) (lesion area) \(\times\) (distance; 75 µm)]. The aortic root was embedded in paraffin and both lesion area and vessel area were calculated from four consecutive 5 µm sections 40 µm apart that cover the entire root of the aorta; the volume fraction of the lesion was calculated as the lesion volume [\(\Sigma\) (lesion area including valve area) \(\times\) (distance; 40 µm)] divided by vessel volume [\(\Sigma\) (vessel area) \(\times\) (distance; 40 µm)] as described\(^3\). All analyses were performed without knowledge of the tissue source.

Oil Red O staining of aortae

PFA-fixed aortae were carefully cleaned from all adventitial fat, washed in isopropanol/H\(_2\)O (2:1) for 5 min and stained with 0.3% Oil Red O for 90 min in 24 well plates at RT. After two washes with isopropanol/H\(_2\)O (2:1) for 2 min, the aortae were transferred to 24-well plates and stored in PBS at 4°C. For en face quantification of lipids, the aortae were placed on a
black silicone plate and cut open starting at the lesser curvature. The aortae were then pinned down with acupuncture needles, and pictures were taken with a Nikon DS-2Mv camera system. Lipid-containing plaque area was determined as percent of Oil Red O-stained area from the total aortic surface area using the AxioVision 4.6 software.

**Immunohistochemistry**

Immunohistochemistry was performed on sections adjacent to that used for quantification of lesion area and using the following antibodies: mouse anti-human α smooth muscle-actin (αSMA, Dako, Glostrup, Denmark), rat anti-mouse Mac-2 (Cedarlane Laboratories, Hornby, Canada) and rabbit anti-human sphingosine kinase 1 (a kind gift from Dr. Lina Obeid, Medical University of South Carolina). Primary antibodies were incubated for 1 hour at room temperature in 3% serum matched to the species of the secondary antibodies followed by biotinylated secondary antibodies for 30 minutes and horseradish peroxidase-conjugated streptavidin for 45 minutes. Visualization was performed with diaminobenzidine and counterstaining of nuclei with haemalaune. Collagen content was assessed using picrosirius red staining and polarized light. Immunostaining for every marker was performed in a single procedure for all sections. An isotype-matched antibody was used on adjacent sections as a negative control. Automated colour segmentation of images was performed and a colour threshold of chromogen stain was defined using the negative control, above which the stain was considered positive (Zeiss AxioVision Rel. 4.6 software). The area containing all positive pixels (µm²) was expressed as percentage of total plaque area.

**Flow cytometry of single aortae**

Single cell suspensions from the aorta were prepared following a protocol graciously provided by Prof. Klaus Ley, Division of Inflammation Biology, La Jolla Institute for Allergy & Immunology, as previously described with minor modifications as to the detection of
apoptotic cells. Briefly, mice were perfused with 0.02 M EDTA through the left ventricle, and the carefully dissected aorta was cut into small pieces and digested in an enzyme cocktail (450U collagenase Type I, 250U collagenase Type XI, 120U hyaluronidase type I-S; all from Sigma, Hamburg, Germany) and 120U DNase I (Roche, Mannheim, Germany) for 60 min at 37°C. The digested tissue was sent through 70 µm cell strainers, pelleted and incubated in 10% FCS/RPMI for 30 min at 37°C. The cells were washed and stained using CD45-PE-Cy7 (BD, Heidelberg, Germany), CD11b-APC (clone M1/70 (or Mac-1) as used in⁴; Milteny, Bergisch Gladbach, Germany) and I-A<sup>b</sup>-PE (BD, Heidelberg, Germany). Annexin-V-FITC (Beckman Coulter, Krefeld, Germany) served to differentiate living from apoptotic cells. Cells negative for Annexin-V were backgated to CD45 and examined for surface expression of CD11b and I-A<sup>b</sup>. Percentage and total number of Annexin-V<sup>-</sup>CD45<sup>+</sup>I-A<sup>b</sup><sup>-</sup>CD11b<sup>+</sup> cells from all CD11b<sup>+</sup> cells was determined quantitatively by measuring the entire cell suspension. Each aorta was assessed individually.

**Macrophage migration assay**

Migration of macrophages was measured in a modified Boyden chamber assay. The upper wells of the chamber were loaded with thioglycollate-elicited peritoneal macrophages (freshly isolated at day 4 after injection of thioglycollate), and the lower wells were supplemented with 1 µM S1P (Biomol, Hamburg, Germany). Migration was quantified after 4 h incubation at 37°C and 5% CO<sub>2</sub>. Cells that had not migrated were removed from the top side of the membrane, and staining was performed with a Hemacolor fast staining Kit (VWR International, Darmstadt, Germany). Migration was quantified from at least three experiments performed in triplicate as indicated.
MCP-1 assay

The MCP-1 Assay (ElisArray mouse MCP-1, SABiosciences, Frederick, MD, USA) was performed using peritoneal lavage fluid according to the manufacturer’s protocol.

Smooth muscle cell proliferation and migration

SMC were isolated from aortae of wild type and S1P$_3^{-/-}$ mice (6-7 aortae per preparation) by enzymatic digestion as described$^5$ and used for experiments up to the 6$^{th}$ passage. Briefly, aortae were incubated in digestion mix (2 mg/ml BSA, 1 mg/ml collagenase 3, 0.375 mg/ml soybean trypsin inhibitor and 0.125 mg/ml elastase type III in DMEM) for 30 min at 37$^\circ$C, after which the adventitia was peeled off, the aortae cut into small pieces and incubated in digestion mix for another 3 hours at 37$^\circ$C. Cells were then plated on Vitrogen100 (Celtrix, Landsdale, USA)-coated tissue culture dishes in 15% fetal calf serum (FCS)/DMEM with 2 mM glutamin and 1 mM pyruvate. For proliferation studies, SMC were serum starved for 24 hours and cultured in 1% FCS in the presence or absence of 1 $\mu$m S1P and 1 nm PDGF, respectively, added every other day for 4 days as described$^6$. Total cell numbers per dish were determined using a Z2 cell counter (Beckmann Coulter, Krefeld, Germany) and proliferation was expressed as percent of that of unstimulated cells. All experiments were performed in triplicate. For migration studies, cells were starved for 24 hours in 1% FCS and seeded in DMEM with 0.25% BSA at a density of 10.000 per well on Vitrogen100-coated membranes in the upper well of a modified Boyden chamber. Migration to 1 $\mu$m S1P, 1 nm PDGF and both, respectively, was assessed after 4 hours of incubation at 37$^\circ$C in 4% CO$_2$ as described$^6$ by counting the cells migrated to the lower side of the membrane in one field of view at 10x magnification (after methanol fixation and staining with a Hemacolor Kit; Merck, Darmstadt, Germany). All migration experiments were performed in triplicate, and five independent experiments were performed.
**Carotid ligation studies**

The left carotid artery was surgically exposed and ligated at the bifurcation as previously described. The whole carotid artery was harvested 4 weeks after the surgery, and serially sectioned over its entire length. Every 10th section starting at the ligation site was stained with haemalaune and eosine. Neointimal and medial areas were quantified by histomorphometry using the AxioVision 4.6 Software (Carl Zeiss Vision GmbH) and the cells in the neointima were counted on each section.

**Statistical Analysis**

Each experiment was performed at least three times as indicated in the figure legends, and data are presented as mean ± SEM. Statistical significance was evaluated by a nonparametric Wilcoxon-Mann-Whitney u-test. Two-sided probability values less than 0.05 were considered significant.

Supplemental figure legends

Figure I. S1P3 deficiency does not alter myeloid cells distribution and content in the bone marrow. A, Bone marrow cells of ApoE−/− and S1P3−/−/ApoE−/− mice on normal chow for 45 weeks were stained for CD11b, Ly-6G and Gr-1 and analyzed by flow cytometry. Myeloid cells were identified with CD11b and further subdivided into neutrophils (CD11b−Ly-6G+), and Ly-6C^high^ and Ly-6C^low^ monocytes (CD11b+Ly-6G^−^Gr-1^+^ and CD11b+Ly-6G^−^Gr-1^−^, respectively). Representative dot plots and histograms are shown. B, Total myeloid cell counts per femur of ApoE−/− and ApoE−/−/S1P3−/− mice shown as mean ± SEM (n = 6 each; n.s. = not significant).

Figure II. Quantification of macrophages in the peritoneum. Thioglycollate-elicited peritoneal macrophages were collected at day 4 after injection and analyzed using flow cytometry. Macrophages were quantified using A, F4/80 and Gr-1 (F4/80^−^Gr-1^−^) and B, CD11b and Ly-6G (CD11b^−^Ly-6G^−^). Representative density plots are shown.

Figure III. Peritoneal macrophage recruitment in S1P3^−^ (donor)→C57BL/6 (recipient) and C57BL/6 (donor)→S1P3^−/−^ (recipient) bone marrow chimera. Peritoneal macrophages were isolated 96 hours after i.p. injection of thioglycollate 10 weeks after bone marrow transplantation. Macrophage numbers were calculated by multiplying the percentage of F4/80^−^Gr-1^−^ cells as determined by flow cytometry with the total number of cells in the peritoneal exsudate. n.s. = not significant.
**Supplemental figure I**

**A** Bone marrow

- **ApoE/−**
- **ApoE/−/S1P3−/−**

**Parameter**
- Total BM cells [10^6/femur]
- Myeloid cells [10^6/femur]
- Neutrophils [10^6/femur]
- Gr-1+(Ly-6Chigh) monocytes [10^6/femur]
- Gr-1−(Ly-6Clow) monocytes [10^6/femur]

<table>
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<th>Parameter</th>
<th>ApoE/− (n=6)</th>
<th>S1P3−/−/ApoE/− (n=6)</th>
<th>p value</th>
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<tr>
<td>Total BM cells [10^6/femur]</td>
<td>24.60 ± 1.51</td>
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<td>n.s.</td>
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<td>Myeloid cells [10^6/femur]</td>
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<td>11.21 ± 1.18</td>
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<tr>
<td>Neutrophils [10^6/femur]</td>
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<td>Gr-1+(Ly-6Chigh)</td>
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<td>2.91 ± 0.36</td>
<td>n.s.</td>
</tr>
<tr>
<td>Gr-1−(Ly-6Clow)</td>
<td>1.26 ± 0.11</td>
<td>1.03 ± 0.22</td>
<td>n.s.</td>
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Supplemental figure II
Peritoneal macrophage recruitment in BM chimera

Supplemental figure III