Sphingosine-1-Phosphate Receptor 3 Promotes Neointimal Hyperplasia in Mouse Iliac-Femoral Arteries

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Objective—The objective of this study was to define a role for sphingosine-1-phosphate receptor 3 (S1PR3) in intimal hyperplasia.

Methods and Results—A denudation model of the iliac-femoral artery in wild-type and S1PR3-null mice was used to define a role for S1PR3 in the arterial injury response because we found in humans and mice that expression of S1PR3 was higher in these arteries compared with carotid arteries. At 28 days after surgery, wild-type arteries formed significantly larger lesions than S1PR3-null arteries. T Bromodeoxyuridine labeling experiments demonstrated that on injury, wild-type arteries exhibited higher medial as well as intimal proliferation than S1PR3-null arteries. Because S1PR3 expression in vitro was low, we expressed S1PR3 in S1PR3-null smooth muscle cells (SMCs) using retroviral-mediated gene transfer to study the effects of S1PR3 on cell functions and signaling. SMCs expressing S1PR3, but not vector-transfected controls, responded to sphingosine-1-phosphate stimulation with activation of Rac, Erk, and Akt. SMCs expressing S1PR3 also migrated more.

Conclusion—In humans and mice, S1PR3 expression was higher in iliac-femoral arteries compared with carotid arteries. S1PR3 promoted neointimal hyperplasia on denudation of iliac-femoral arteries in mice, likely by stimulating cell migration and proliferation through activation of signaling pathways involving Erk, Akt, and Rac. (Arterioscler Thromb Vasc Biol. 2012;32:00-00.)

Key Words: receptors ■ restenosis ■ signal transduction ■ vascular biology ■ sphingosine-1-phosphate

Sphingosine-1-phosphate (SIP) is a bioactive sphingolipid that plays a role in vascular pathologies, including coronary artery disease,1 atherosclerosis,2–6 and intimal lesion formation after arterial injury.7–5 S1P is present at submicromolar concentrations in plasma, where it is bound mainly to albumin and high-density lipoproteins.10 To what extent blood-borne S1P is recognized by vascular cells in normal arteries is unknown. In diseased arteries or on arterial injury, SIP may be released from platelets and also locally produced by growth factor or cytokine-mediated induction of sphingosine kinase, which phosphorylates sphingosine to produce by growth factor or cytokine-mediated induction of sphingosine kinase, which phosphorylates sphingosine to generate S1P.11 S1P binds to 5 G protein-coupled receptors, S1PR1 to S1PR5. S1PR1, S1PR2, and S1PR3 are ubiquitously expressed, whereas S1PR4 and S1PR5 are mainly expressed in immune cells and brain, respectively.12

Restenosis is a significant complication of surgical and percutaneous procedures to restore arterial blood flow. Arterial lesions are characterized by intimal hyperplasia and negative remodeling of the artery, both causing luminal narrowing. In animal models of neointimal formation, injury-induced medial proliferation is followed by an accumulation of intimal cells.13,14 The role for S1P in arterial lesion formation may be complex because cellular responses to S1P depend on which receptor is expressed, given that they couple to different G-proteins.12 We have recently demonstrated that S1PR2 is an inhibitor of neointimal lesion growth induced by carotid ligation by comparing the injury response in wild-type and S1PR2-null mice.15 This observation is in agreement with in vitro experiments showing that S1PR2 mediates the inhibition of smooth muscle cell (SMC) migration15–17 and the induction of genes of the differentiated SMC phenotype.7,8 In the rat carotid artery, application of an antagonist for both S1PR1 and S1PR3 reduces intimal lesion size following denudation injury.9 S1PR1 is also required for recruitment of mural cells to the developing endothelial tube,19 so it may promote injury-induced intimal hyperplasia by stimulation of medial cell migration. Only recently has the role of S1PR3 in intimal hyperplasia been addressed. During the preparation of this article, it was reported that deletion of S1PR3 increases lesion size in carotid arteries following
ligation injury. Here, we report that deletion of S1PR3 has the opposite effect in denuded iliac-femoral arteries, in which it decreases neointimal lesion size. Possible reasons for S1PR3 playing opposing roles in the arterial injury response using different models are discussed.

Methods

Please see the online-only Data Supplement for more detailed descriptions of methods.

Animals and Human Tissue

S1PR3-null mice and wild-type mice were kindly provided by Dr Richard L. Proia (National Institutes of Health, Bethesda, MD).20 Heterozygous mice were bred to generate S1PR3-null and wild-type littermates. Genotypes were verified by polymerase chain reaction analysis using specific primers (online-only Data Supplement). To obtain human specimens, patients with arterial occlusive disease in the carotid or iliac-femoral artery were recruited. Tissue was taken from the artery around the site of the incision or disobliteration and immersed immediately in RNAlater (Qiagen). This study was conducted in accordance with rules and regulation of the ethics commission of the General Medical Council, Hamburg, Germany.

Iliac-Femoral Artery Denudation

Adult male mice were anesthetized with an intraperitoneal injection of xylazine and ketamine cocktail (8.8 mg/kg xylazine and 130 mg/kg ketamine). The popliteal artery was exposed, and 2 ligatures (6-0 surgical silk) were placed around it. The artery was dilated by local administration of 1% lidocaine, and the distal ligature was tied. A small incision was then made between the 2 ligatures, and the vessel was denuded by using a 7-0 monofilament loop catheter. At the appropriate time point, mice were euthanized with Beuthanasia-D (500 mg/kg, IP), and iliac-femoral arteries were perfusion-fixed with 4% paraformaldehyde, embedded in paraffin. Measurements of cross-sectional areas were performed on tissue sections following staining with hematoxylin and eosin using computer-assisted image analysis (National Institutes of Health Image).

For analysis of proliferating cells, mice were intraperitoneally injected with bromodeoxyuridine (BrdU) (30 mg/g body weight) at 1, 9, and 17 hours before euthanasia. Tissue sections were stained with BrdU antibody (Roche) and hematoxylin. Hematoxylin-positive (total) and BrdU-positive (replicating) cells were counted. All studies were performed in accordance with the guidelines for animal experimentation at the National Institutes of Health and at the University of Washington.

Analysis of Gene Expression by Real-Time Polymerase Chain Reaction

Real-time polymerase chain reaction was performed as previously described.7 Specificity of primers was verified by dissociation curves, and gene expression was normalized to expression of glyceraldehyde-3-phosphate dehydrogenase.

Isolation and Culture of Mouse Carotid SMCs

Carotid SMCs were prepared and cultured as previously described.21

Expression of S1PR3 in SMCs Using Retroviral-Mediated Gene Transfer

Human S1PR3 cDNA was kindly provided by Timothy Hla (Cornell University, New York, NY) and LXSH vector by Dusty Miller (Fred Hutchinson Cancer Research Center, Seattle, WA). S1PR3 cDNA was subcloned into the BamHI site of the LXSH retrovirus backbone vector. PE501 early high-titer ecotropic packaging cells were transfected with S1PR3-LXSH plasmid using Lipofectamine-2000 transfection agent (Invitrogen). Viral particles in cell supernatant were used to infect a high-titer PA317 amphotropic packaging cell line. This step was performed twice to increase titers. Several clones were probed for S1PR3 expression and used to produce virus to transfect S1PR3-null carotid SMCs. Multiple SMC clones were probed for S1PR3 expression, and clones strongly expressing S1PR3 were selected and expanded for experiments (S1PR3-SMCs). SMC clones expressing empty vector were used as controls (LXSH-SMCs).

Rac and RhoA Assays

Activities of Rac1 and RhoA were measured using commercially available kits (Rac G-LISA and RhoA G-LISA, both from Cytoskeleton).

cAMP Measurements

cAMP was measured using cAMP Biotrak enzyme-immunooassay system (GE Healthcare) per the manufacturer’s instructions.

Analysis of Phospho-Erk and Phospho-Akt by Western Blotting

Blots were probed with phospho-Erk and phospho-Akt (T308) antibodies overnight at 4°C and then developed with enhanced chemiluminescence (GE Healthcare). Equal loading of protein was confirmed by reprobing blots for β-tubulin. All antibodies were from Cell Signaling Technology.

Proliferation Assay

Cell replication was measured using a metabolic labeling assay.24

Scratch Assay

LXSH-SMCs and S1PR3-SMCs were plated into 12-well dishes at 100,000 cells per well. Twenty-four hours later, a vertical scratch was made to the cell monolayer using a sterile 1-mL pipette tip. A perpendicular line was drawn onto the plate to mark a specific location, and a picture was taken. Medium was replaced with serum-free medium, and S1P (Cayman) or platelet-derived growth factor-BB (PDGF-BB) (R&D Systems) was added as indicated. After 24 hours, the scratch was imaged at the same position, and cell-free areas were quantified using Imagej software (National Institutes of Health). Migration was then expressed as percentage of closure of cell-free area.

Statistics

As indicated in figure legends, data are presented as mean±SD or SEM. Significance of differences between 2 groups was calculated using the Student t test. Multiple comparisons using 1 data set were analyzed by applying a Bonferroni adjustment.25 A probability of P<0.05 was considered significant.

Results

Iliac-Femoral Arteries Express Significantly More S1PR3 Than Carotid Arteries

The C57BL/6 mouse is widely used to study the response to arterial injury. We and others have noticed that C57BL/6 mice can be considered resistant to neointimal lesion growth following injury of the carotid artery because injury produces no or only small lesions compared with mice of different genetic backgrounds.13,26,27 In contrast, C57BL/6 iliac-femoral arteries respond to injury with significant neointimal lesion formation.28 One possibility to explain this difference may lie in expression of S1PRs because we found that S1PR3 is strongly expressed in iliac-femoral arteries compared with carotid arteries (Figure 1). Interestingly, this difference has also been observed in humans (Figure 1). We therefore chose denudation of the iliac-femoral artery to investigate a possible role for S1PR3 in neointimal lesion growth.
S1PR3 Promotes Intimal Hyperplasia

To define a role for S1PR3 in the arterial injury response, we compared medial and intimal areas at various time points following denudation of iliac-femoral arteries in wild-type and S1PR3-null mice. Lack of S1PR3 expression decreased neointimal lesion size at 28 days postinjury by 90% (Figure 2C). In contrast, intimal cell numbers continuously increased in wild-type animals throughout the time course (4–28 days) but stayed constant after day 7 in S1PR3-null mice, resulting in a significantly higher number of intimal cells in wild-type animals at day 28 postinjury (Figure 2D). BrdU labeling experiments showed that wild-type medial as well as intimal SMCs proliferated more compared with S1PR3-null at days 7 and 14 postinjury (Figure 3A and 3B). For medial SMCs, this difference was still significant at day 28 postinjury (Figure 3A). The kinetics of proliferation after injury were similar in wild-type and S1PR3-null arteries. Medial proliferation was low at day 4, peaked at day 7, and then declined. Intimal proliferation was the highest at day 4 and then declined (Figure 3A and 3B). These data suggest that in response to injury, S1PR3 expression promotes cell proliferation in the media as well as in the developing intima. We also measured expression of S1PRs at 4 and 7 days after injury. Unexpectedly, expression of S1PR3 decreased to only 10% compared with unjured controls at day 4 postsurgery but then recovered to 25% at day 7 (Figure 1 in the online-only Data Supplement). In both wild-type and S1PR3-null mice, injury stimulated expression of S1PR1 (at day 7) and S1PR2 (at day 4 and day 7). To address the question of whether S1PR3 affects the inflammatory response to injury, we measured expression of the common leukocyte antigens CD45 and CD14, markers for monocytes, macrophages, and dendritic cells. There was no difference in expression levels of CD45 or CD14 between wild-type and S1PR3-null arteries. Expression of both markers was induced by injury (approximately 10-fold at day 4) but with different kinetics in that CD45 expression further increased between day 4 and day 7, whereas CD14 expression did not (Figure II in the online-only Data Supplement).

S1PR3 Activates Rac, Akt, and Erk but Does Not Inhibit S1PR2-Dependent Activation of Rho

To define S1P-induced signaling pathways in SMCs that are mediated by S1PR3, we attempted to prepare SMCs from iliac-femoral arteries. However, we never succeeded in growing sufficient numbers of cells to perform in vitro experi-

Figure 1. Expression of sphingosine-1-phosphate receptors (S1PRs) in mouse and human carotid and iliac-femoral arteries. Total RNA from carotid and iliac-femoral arteries from C57BL/6 mice and human samples was prepared and subjected to real-time polymerase chain reaction analysis for expression of S1PR1, S1PR2, and S1PR3. A typical cross-section of wild-type and S1PR3–null arteries. Wild-type and S1PR3-null iliac-femoral arteries were denuded and injected with bromodeoxyuridine (BrdU) (30 μg/g body weight) at 17 hours, 8 hours, and 1 hour before euthanization at the indicated time points. Arteries were perfusion fixed, and tissue sections stained with anti-BrdU (Roche). The numbers of positive cells in media (A) and intima (B) were quantitated by counting multiple sections that were 250 μm apart. Data (mean±SEM, n=7–13 animals per group) are presented as percentage of BrdU-positive cells. *P<0.05.

Figure 2. Sphingosine-1-phosphate receptor 3 (S1PR3) promotes intimal hyperplasia. Wild-type and S1PR3-null iliac-femoral arteries were denuded and were euthanized at the indicated time points. Arteries were perfusion fixed, and tissue sections were stained with hematoxylin/eosin. A, For the 28-day time point, intimal areas were measured using National Institutes of Health Image software. Data are shown as mean±SEM for wild-type (n=8 for human iliac-femoral arteries, and n=5 for human carotid arteries) are presented as percentage of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression. **P<0.001. B, A typical cross-section of wild-type and S1PR3-null arteries at 28 days after injury is shown. Arrowheads mark the external and internal elastic lamina. Scale bars=100 μm. The number of cells in media (C) and intima (D) was quantitated by counting multiple sections that were 250 μm apart. Data (mean±SEM, n=7–13 animals per group) are presented as number of cells counted per section. *P<0.05.
ments. We then prepared SMCs from carotid arteries derived from wild-type and S1PR3-null mice and measured S1PR1, S1PR2, and S1PR3 message levels. We included NIH3T3 cells as a potential positive control for S1PR3 expression because fibroblasts may express more S1PR3 than SMCs.29,30 Indeed, compared with NIH3T3 cells, wild-type SMCs expressed significantly less S1PR1 and S1PR3 (Figure 4A), whereas S1PR2 expression was similar in both cell types.
Consistent with higher expression of S1PR1 and S1PR3, only NIH3T3 cells, and neither wild-type nor S1PR3-null SMCs, responded to S1P with phosphorylation of Erk and Akt (Figure 4B, negative data for SMCs not shown). We therefore decided to overexpress S1PR3 in S1PR3-null carotid SMCs to investigate the possibility that high expression of S1PR3 in iliac-femoral arteries stimulates Erk/Akt-dependent signaling pathways. We used retroviral gene transfer to generate multiple SMC clones strongly expressing S1PR3 (S1PR3-SMCs). Carotid S1PR3-null SMCs were also transfected with empty vector (LXSH-SMCs) and used as controls.20 There was no significant difference in expression of S1PR1 or S1PR2 between LXSN- and S1PR3-SMCs (Figure 4A). Compared with NIH3T3 cells, S1PR3-SMCs expressed approximately 5 times as much S1PR3 message (Figure 4A). Following stimulation with S1P, only S1PR3-SMCs but not LXSH-SMCs showed phosphorylation of Erk and Akt (Figure 4B). As S1P positively regulates cell migration by activating Rac via S1PR1 and S1PR3, or negatively by activating Rho via S1PR2,16,17 we measured S1P-induced activation of Rac and Rho in S1PR3-SMCs and LXSN controls. We found that Rac activation by S1P was S1PR3 dependent and that S1PR3 did not affect activation of Rho by S1P2 (Figure 4C and 4D). To address the question of whether S1PR1 is signaling, we measured the effect of S1P on forskolin-induced cAMP levels. As S1PR1 couples to Goi, S1P addition should reduce cAMP levels if S1PR1 is active. Forskolin led to increased cAMP levels in all cells (Figure 4E). Addition of S1P, however, reduced cAMP levels only in cells expressing S1PR3 (wild-type SMCs, S1PR3-SMCs, and NIH3T3 cells), not in cells lacking S1PR3 (S1PR3-null SMCs and LXSN controls; Figure 4E). This observation indicates that S1PR1 does not contribute to S1P responses in cultured SMCs.

**S1PR3 Promotes Proliferation and Migration of SMCs**

Because injury-induced neointimal lesion formation was significantly decreased in S1PR3-null arteries compared with wild-type controls, we tested the possibility that S1PR3 promotes SMC proliferation or migration. Cell proliferation was assessed by using a metabolic labeling assay in which mitochondrial dehydrogenase activity is correlated to cell numbers.24 Measurements of mitochondrial dehydrogenase activity indicated that there were more S1PR3-SMCs than LXSH-SMCs at all time points after serum stimulation, suggesting that S1PR3 promotes cell growth (Figure 5A). Cell migration was measured using scratch assays, and migration was expressed as percentage of “wound” closure. As expected from previous reports showing that S1P2 is an inhibitor of migration,15,31 S1P inhibited PDGF-BB-induced migration of LXSN-SMCs (P<0.05, n=3, PDGF-BB versus PDGF-BB+S1P), and S1P alone did not stimulate migration (Figure 5B). In contrast, in SMCs overexpressing S1PR3, S1P itself stimulated migration (P<0.05, n=3, control versus S1P). Moreover, S1PR3 expression appeared to abolish the inhibitory effect of S1PR2 as in S1PR3 SMCs, and it increased migration even in the presence of PDGF-BB (P<0.05, n=3 PDGF-BB versus PDGF-BB + S1P; Figure 5B). LXSH-SMCs and S1PR3-SMCs migrated equally well in presence of PDGF-BB, indicating that S1PR3 expression had no general effect on cell migration (Figure 5B). In the presence of S1P, however, migration of S1PR3-SMCs was always greatly increased compared with LXSN-SMCs (Figure 5B).

**Discussion**

The role of S1P in biological processes is complex because cells may express multiple S1PRs that couple to different G-proteins, which may mediate different, even opposing effects on cells. It has previously been shown that concomitant pharmacological inhibition of S1PR1 and S1PR3 attenuates lesion formation in the rat carotid artery denudation model, suggesting a positive role for these receptors in intimal hyperplasia.9 Genetic deletion of S1PR2 increases lesion size in mouse carotid arteries following ligation.15 The main reason that we chose the iliac-femoral artery as a model to define a role for S1PR3 in arterial stenosis was that S1PR3 expression in mouse iliac-femoral arteries is much higher than in carotid arteries (Figure 1). Interestingly, this difference was also found in humans and may indicate the possibility of targeted pharmacological treatment to inhibit restenosis in iliac-femoral arteries by using S1PR3 antagonists. Following denudation of the iliac-femoral artery, wild-type mice developed much larger lesions than S1PR3-null mice, and this correlated with higher medial as well as intimal proliferation in wild-type arteries (Figures 2 and 3). Notably, despite higher medial proliferation in wild-type arteries compared with S1PR3-null arteries, medial cell numbers did not significantly differ in the 2 arteries, whereas intimal cell numbers were higher in wild-type arteries. We therefore believe that proliferating medial cells migrate into the intima where they contribute to neointimal lesion formation. Given that S1PR3 expression promotes neointimal lesion formation, we were surprised to find that injury caused a downregulation of S1PR3 expression (Figure 1 in the online-only Data Supplement). It is possible that protein levels were less affected by injury than RNA levels, but we cannot comment on receptor protein levels because we have not found an antibody that detects endogenous S1PR3 levels in the vascu-
lature. Nevertheless, genetic deletion of S1PR3 strongly inhibited neointimal lesion formation, and we conclude that the receptor is functional despite decreased expression after injury. Given the recent observation that S1PR3 regulates the macrophage content in atherosclerotic lesions in apolipoprotein E–null mice and that S1P is critical for leukocyte egression from lymphatic organs, we tested the possibility that S1PR3 affects the inflammatory response to injury by measuring the expression of the common leukocyte antigen, CD45, and CD14, a marker for macrophages, monocytes, and dendritic cells. As expected, we found increased arterial expression of both CD14 and CD45 after injury, but there was no difference between wild-type and S1PR3-null arteries (Figure II in the online-only Data Supplement), suggesting that S1PR3 does not play a role in recruiting or retaining inflammatory cells in this model.

Our data demonstrate that in carotid wild-type SMCs, S1PR3 reduced forskolin-induced cAMP levels (see Figure 4E) but did not promote activation of Erk or Akt (data not shown). SMCs expressing high levels of S1PR3, however, responded to S1P with activation of Erk and Akt. One possible explanation is that different thresholds exist for activation of these pathways. Another possibility is that S1PR3, which primarily couples to Gq, may at high receptor densities also couple to Gαi, a strong activator of PLCβ, which mediates activation of Erk and Akt. Consistent with S1PR3-mediated activation of Erk and Akt is our observation that SMCs overexpressing S1PR3 grew faster and migrated better than S1PR3-deficient controls (Figure 5). Notably, S1P no longer inhibited PDGF-BB-induced migration in S1PR3-overexpressing cells (Figure 5B). As S1P-mediated inhibition of cell migration depends on S1PR2-dependent activation of Rho,15,31 we tested the possibility that S1PR3 expression inhibits Rho activation by S1P. As this was not the case (Figure 4D), S1PR3-mediated hyperactivation of Erk and Akt may simply counteract the inhibitory function of S1PR2-dependent activation of Rho. Thus, expression ratios between S1PR2 and S1PR3 (and possibly S1PR1) may be more indicative of the formation of neointimal lesions in iliac-femoral arteries than absolute expression levels of any individual S1PR.

While our studies were in progress, it was reported that S1PR3-null mice develop larger lesions in ligated carotid arteries than wild-type animals, suggesting an inhibitory role for S1PR3 in intimal hyperplasia. There are several possible reasons for opposite effects of S1PR3 in iliac-femoral arteries (this article) and carotid arteries. One possibility is that there are colony-specific properties, because the mice used by us and Dr Levkau’s laboratory stem from different sources. To address this important point, we performed carotid ligation injury in our wild-type and S1PR3-null mice. These experiments confirmed the observations made by Dr Levkau’s group that S1PR3-null animals develop significantly larger lesions at 4 weeks after injury (Figure III in the online-only Data Supplement). Another explanation for the opposing roles of S1PR3 is that in carotid arteries, where S1PR3 expression is low, S1PR3 functions mainly to inhibit production of cAMP, long known to be an inhibitor of SMC growth. Thus, deletion of S1PR3 in these arteries might stimulate neointimal lesion growth. In contrast, in iliac-femoral arteries, S1PR3 is highly expressed and induces activation of Erk, Rac, and Akt, all of which are known to promote cell growth and migration.8–41 Thus, deletion of S1PR3 might reduce neointimal lesion formation. Finally, another possibility is that S1PR3 inhibits lesion growth in carotid arteries because of its function in endothelial cells, where S1PR3 is anti-inflammatory by activating endothelial nitric oxide synthase through Akt-dependent phosphorylation.35–37 Endothelial cells are present in the ligated carotid artery but are removed from the iliac-femoral artery by the denudation procedure. In agreement with a lesion-inhibitory role for S1PR3 through endothelial nitric oxide synthase activation is the observation that endothelial nitric oxide synthase–deficient mice grow much larger lesions following carotid ligation compared with wild-type controls.38

In conclusion, function of S1PR3 in the arterial injury response depends on the vascular bed and possibly expression levels and type of injury. Therefore, pharmacological approaches directed against S1PR3 to block restenosis in iliac-femoral arteries might have a negative impact on restenotic lesions in other vascular beds. Moreover, our finding that expression differences regarding S1PR3 also exist in humans indicates the possibility of artery-specific pharmacological treatment of restenosis.

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Disclosures
None.

References


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Genotyping of S1PR3 mice

RedExtract-N-Amp Tissue PCR Kit (Sigma) was used to extract DNA from ear punches obtained from wild-type and S1PR3-null mice. In standard PCR assays, primers 1 and 2 detect the S1PR3 wild-type allele, primers 2 and 3 the S1PR3-null allele.

Primer 1: 5'-TCAGTATCTTCACCGCCATT-3'
Primer 2: 5'-AATCACTACGGTCCGCAGAA-3'
Primer 3: 5'-GTGCAATCCATCTTTGTTCAAT-3'

Iliac-femoral artery denudation

Adult male mice were anesthetized with an intraperitoneal injection of xylazine and ketamine cocktail (8.8 mg/kg xylazine and 130 mg/kg ketamine). To ensure an adequate anesthetic plane in the mice, we monitored corneal reflexes, respiration and signs of flinching using the front toe pinch method. The popliteal artery, a branch of the left femoral artery, was exposed between the two muscles, and then two ligatures (6-0 surgical silk) were placed around it. The artery was dilated by local administration of 1% lidocaine and the distal ligature was tied. After blood flow was controlled with the proximal ligature, a small incision was made between the two ligatures and a denudation device (7-0 monofilament loop catheter) was introduced into the left femoral artery and pushed up to the aortic bifurcation. The catheter was withdrawn with constant rotation to remove the endothelium. This procedure was repeated three times before
removing the catheter. The popliteal artery was then ligated, and the skin incision was closed with wound clips. At the appropriate time point, mice were euthanized with Beuthanasia-D (500 mg/kg, i.p.) and iliac-femoral arteries were perfused with 4% paraformaldehyde via a catheter placed in the thoracic aorta. Iliac-femoral arteries were removed and then divided into two pieces at their center, embedded in paraffin and 3 sections (50 \( \mu \)m apart) were cut from each segment. Measurements of cross sectional areas were performed on tissue sections following staining with hematoxylin and eosin using computer-assisted image analysis (NIH image).

For analysis of proliferating cells, mice were intraperitoneally injected with bromodeoxyuridine (BrdU, 30 mg/g body wt) at 1, 9, and 17 hours before euthanasia. Tissue sections (see above) were stained with BrdU antibody (Roche) and hematoxylin. Hematoxylin-positive (total) and BrdU-positive (replicating) cells were counted. All studies were performed in accordance with the guidelines for animal experimentation at the NIH and at the University of Washington.

**Carotid artery ligation injury**

Adult male S1PR3-null mice and littermate wild-type mice were anesthetized as described above. A midline incision on the ventral neck was made, and the connective tissue and nerve were cleaned from contact with the distal portion of the carotid artery. A suture loop (6-0 surgical silk) was placed just below the external/internal bifurcation and the common carotid artery was ligated. The skin incision was closed with wound clips.
Rac and RhoA assay

Activities of Rac1 and RhoA were measured using commercially available kits (Rac G-LISA, RhoA G-LISA, both from Cytoskeleton). LXSH-SMCs and S1PR3-SMCs were plated into 10 cm dishes with 10% FBS (600,000 cells/plate) and serum-starved the next day for 48-72 hours. Cells were stimulated with S1P (1 µmol/L) for 5 min, then washed twice with ice-cold PBS, and scraped into lysis buffer (Cytoskeleton). Activated Rac1 and RhoA were quantified following the manufacturer’s protocol. A positive control provided by the kit was included. Relative activities of Rac1 and RhoA were calculated by subtracting background absorption from experimental values and normalizing data to activities obtained with quiescent wild-type cells.

Cyclic AMP measurements

Cells (10,000/well) were in 96-well plates in media containing 10% FBS. Next day, cells were stimulated for 5 min with S1P (1 µmol/L) and forskolin (3 µmol/L, Sigma) as indicated in the figure legend. Cyclic AMP was measured using cAMP Biotrak enzyme-immunoassay system (GE Healthcare) as to the manufacturer’s instructions.

Analysis of phospho-Erk and phospho-Akt by Western blotting

SMCs were plated into 6-well plates with 10% FBS (100,000 cells/well) and media was changed the next day to serum-free media. After 2-3 days, cells were stimulated as indicated in figure legends and extracted with Laemmli sample buffer. Total cell lysates (equal amounts) were then subjected to SDS-polyacrylamide gel electrophoresis.
followed by Western blotting. Blots were probed with phospho-Erk and phospho-Akt (T308) antibodies over night at 4°C and then developed with ECL (GE Healthcare).
Equal loading of protein was confirmed by re-probing blots for β-tubulin. All antibodies were from Cell Signaling Tech.

**Proliferation Assay**
Cell replication was measured using a metabolic labeling assay.²³ LXSH-SMCs and S1PR3-SMCs were plated into growth media containing 10% FBS at 6,000 cells per well into12 well dishes. At the appropriate time points, cells were incubated with 5 mg/mL MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma) at 37°C for one hour. Cells were then washed with PBS, dried, and MTT precipitate (formazan) was solubilized in 0.1 mL DMSO/well. Absorption of MTT solution was measured at 560 nm and corrected for DMSO alone.

**Analysis of gene expression by real time PCR**
Total RNA was prepared from human tissue samples or pools of frozen mouse carotid (8-10) or iliac-femoral arteries (10-12) using RNeasy Fibrous Tissue Mini Kit (Qiagen). RNA (1 µg) was reverse transcribed using Superscript II RNAse H- Reverse Transcriptase (Invitrogen). cDNA (100-200 ng/reaction) was combined with Sensimix (Quantace) and specific primers (see below). Cycle threshold numbers were determined with an ABI 7500 thermocycler (Applied Biosystems) using 40 cycles (95°C, 15 sec;
60°C, 1 min). Specificity of primers was verified by dissociation curves, and gene expression was normalized to expression of GAPDH.

**Primers used for real time PCR**

The same primers were used for mouse and human genes.

**CD14**

5’-CTCTGTCCCTAAAGCGGCTTAC-3’
5’-GGTGGAGTCAAGATTTGTT-3’

**CD45**

5’CAGAAACGCTAAGCCTAGTTG-3’
5’ATGCAGGATCAGGTTTAGATGC-3’

**GAPDH**

5’-TCCTGCACCACAACTGCTT-3’
5’-AGGGGCATCCACAGTCTTC-3’

**S1PR1**

5’-ATCATGGGCTGGAACTGCATCA-3’
5’-CGAGTCCTGACCAAGGAGTAGAT-3’

**S1PR2**

5’-CAGACGCTAGCCCTGCTCAAGA-3’
5’-TAGTGCTCTTTGTAGAGGA-3’

**S1PR3**

5’-ACAACGCATGTACTTTTCAT-3’
5’-TACTGCCCTCCCTGAGGAACCA-3’

**Isolation and culture of mouse carotid SMCs**

Both carotid arteries from 3-5 adult male mice were excised, cleaned of excess tissue, and placed into enzyme mix (2 mg/mL BSA (Sigma), 1 mg/mL collagenase (Worthington), 0.375 mg/mL soybean trypsin inhibitor (Worthington), and 0.125 mg/mL
elastase type III (Sigma) in Hanks’ balanced salt solution). After 10 minutes of incubation at 37°C, the adventitial layer was removed, and the remaining tissue was further incubated in enzyme mix for 45 min at 37°C. Tissue and cells were then collected by centrifugation and plated in DMEM (GIBCO) supplemented with antibiotics (200 U/mL penicillin, 0.2 mg/mL streptomycin, all from GIBCO) and 10% fetal bovine serum (FBS, Atlantic Biologics). Cells were typically used between passage 5 and 12.

**Supplemental Figures**

**Supplemental Figure I. Injury regulates S1PR expression in iliac-femoral arteries.** Wild-type and S1PR3-null iliac-femoral arteries were denuded and animals sacrificed at the indicated time points. 8-12 arteries were pooled and total RNA was prepared for real time PCR analysis of S1PR expression. Data (mean +/- S.D., N=2) are shown for two independent pools (day 4) and one technical repeat using one pool for day 7.
**Supplemental Figure II. Injury induces expression of CD45 and CD14 in iliac-femoral arteries.** Wild-type and S1PR3-null iliac-femoral arteries were denuded and animals sacrificed at the indicated time points. 8-12 arteries were pooled and total RNA was prepared for real time PCR analysis of S1PR expression. Data (mean +/- S.D., N=2) are shown for two independent pools (day 4) and one technical repeat using one pool for day 7.

**Supplemental Figure III. S1PR3 inhibits ligation-induced neointimal lesion formation in carotid arteries.**

Wild-type and S1PR3-null carotid arteries were ligated as described above. Animals were sacrificed at 28 days post surgery. Arteries were perfusion-fixed, stained with H&E and intimal area was assessed using NIH Image software. Data (mean +/- S.D.) are presented in arbitrary units (N=9 for wild-type, N=8 for S1PR3-null). *P<0.05