Supplemental Material

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 -GTGCAATCCATCTTGTTCAAT-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 The catheter was withdrawn with constant rotation to remove the endothelium.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 μm 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.

<u>Carotid artery ligation injury</u>

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;

$60^{o}C$, 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.

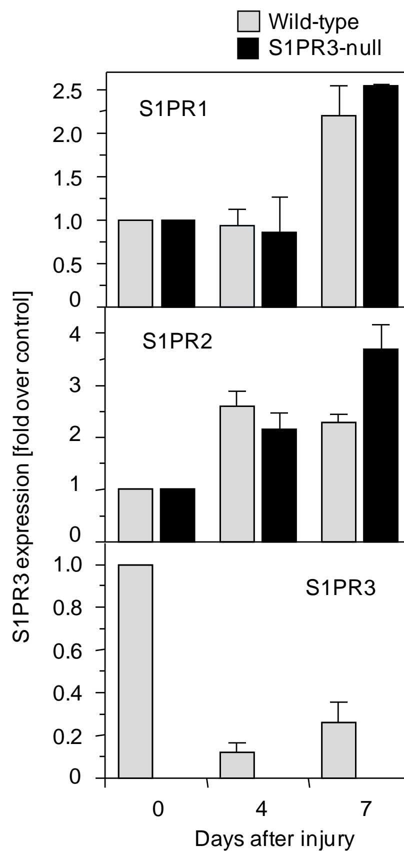
CD14:	5'-CTCTGTCCTTAAAGCGGCTTAC-3'
	5'-GTTGCGGAGGTTCAAGATGTT-3'
CD45	5'CAGAAACGCCTAAGCCTAGTTG-3'
	5'ATGCAGGATCAGGTTTAGATGC-3'
GAPDH:	5'-TCCTGCACCACCAACTGCTT-3'
	5'-AGGGGCCATCCACAGTCTTC-3'
S1PR1:	5'-ATCATGGGCTGGAACTGCATCA -3'
	5'-CGAGTCCTGACCAAGGAGTAGAT-3'
S1PR2:	5'-CAGACGCTAGCCCTGCTCAAGA-3'
	5'-TAGTGGGCTTTGTAGAGGA-3'
S1PR3:	5"-ACAACCGCATGTACTTTTTCAT-3'
	5'-TACTGCCCTCCCTGAGGAACCA-3'

Isolation and culture of mouse carotid SMCs

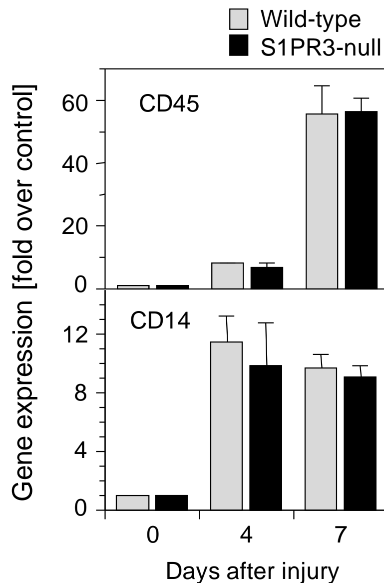
Both carotid arteries from 3-5 adult male mice were excised, cleaned of excess tissue, and placed into enzyme mix (2 mg/mL BSA (Sigma), 1 mg/mL collagenase (Worthington), 0.375 mg/mL soybean trypsin inhibitor (Worthington), and 0.125 mg/mL

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.

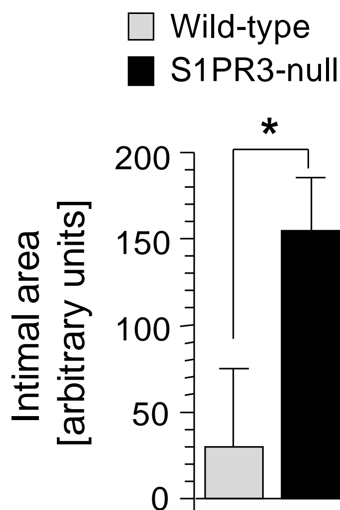
Supplemental Figures



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). $*P<0.05$