Sphingosine-1-Phosphate Prevents Tumor Necrosis Factor-α–Mediated Monocyte Adhesion to Aortic Endothelium in Mice

David T. Bolick, Suseela Srinivasan, Kyu W. Kim, Melissa E. Hatley, Jeremy J. Clemens, Angela Whetzel, Nicole Ferger, Timothy L. Macdonald, Michael D. Davis, Philip S. Tsao, Kevin R. Lynch, Catherine C. Hedrick

Objective—Endothelial activation and monocyte adhesion to endothelium are key events in inflammation. Sphingosine-1-phosphate (S1P) is a sphingolipid that binds to G protein-coupled receptors on endothelial cells (ECs). We examined the role of S1P in modulating endothelial activation and monocyte–EC interactions in vivo.

Methods and Results—We injected C57BL/6J mice intravenously with tumor necrosis factor (TNF)-α in the presence and absence of the S1P1 receptor agonist SEW2871 and examined monocyte adhesion. Aortas from TNF-α–injected mice had a 4-fold increase in the number of monocytes bound, whereas aortas from TNF-α plus SEW2871-treated mice had few monocytes bound (P<0.0001). Using siRNA, we found that inhibiting the S1P1 receptor in vascular ECs blocked the ability of S1P to prevent monocyte–EC interactions in response to TNF-α. We examined signaling pathways downstream of S1P1 and found that 100 nM S1P increased phosphorylation of Akt and decreased activation of c-jun.

Conclusions—Thus, we provide the first evidence that S1P signaling through the endothelial S1P1 receptor protects the vasculature against TNF-α–mediated monocyte–EC interactions in vivo. (Arterioscler Thromb Vasc Biol. 2005; 25:1-6.)

Key Words: PLEASE ■ SUPPLY ■ KEY ■ WORDS ■ XXXX

Inflammation is a hallmark of atherosclerosis and diabetes. Monocyte–endothelial interactions are key initiating events of inflammation. Activated monocytes release tumor necrosis factor (TNF)-α that mediates a variety of pathological vascular responses. Monoclonal antibody therapies to reduce TNF-α have reduced inflammation in several chronic diseases, but these therapies do not prevent the initiation of inflammation.

Sphingosine-1-phosphate (S1P) is a biologically active sphingolipid in circulation that evokes a variety of cellular responses, including cell migration, vascular maturation, and lymphocyte homing. However, the role of S1P in vascular inflammation is unknown. S1P binds to 5 G protein-coupled receptors (Edg receptors) on endothelium and activates these receptors in the low nanomolar range. In the vasculature, S1P receptors are found on both endothelial cells (ECs) and monocytes. S1P1 is involved in endothelial migration and blood vessel formation. S1P3 regulates heart rate in mice and vascular tone through activation of endothelial nitric oxide synthase (eNOS).

In the current study, we report that S1P prevents TNF-α–mediated monocyte adhesion to intact aorta and to cultured primary isolates of aortic endothelial cells. We found that the protective, antiinflammatory action of S1P is mediated through binding to the endothelial S1P1 receptor.

Methods

Detailed Methods for all experiments can be found online. Please see http://atvb.ahajournals.org.

Results

S1P and an S1P1 Receptor Agonist Prevent TNF-Mediated Monocyte–Endothelial Interactions

We injected C57BL/6J (B6) mice with 0.5 μg of recombinant murine TNF-α intravenously, and either 5 mg/kg of the selective S1P1 receptor agonist, SEW2871 (5-(4-Phenyl-5-trifluoromethylthiophen-2-yl)-3-(3-trifluoromethylphenyl)-1,2,4-oxadiazole), or saline plus 0.2% fatty acid-free bovine serum albumin as a vehicle control. SEW2871 is a selective agonist for S1P1 and is ≈30-fold less potent than S1P at S1P1, with no agonist activity at S1P2 or S1P3 at concentrations up to 10 micromolar. At 2 hours after injection, aortas were harvested. Subgroups of aortas received 100 ng/mL pertussis toxin (PTX) to uncouple S1P1/S1P3 Gai receptor
Figure 1. S1P prevents TNF-α-mediated monocyte–endothelial cell interactions in vivo. A, C57BL/6J mice were injected intravenously with saline or 0.2% FAFBSA (saline), or with 500 ng murine TNFα for 2 hours (+TNF), or with 500 ng murine TNFα plus 5 mg/kg SEW2871, a S1P1 agonist for 2 hours (+TNF + SEW2871). All aortas were harvested and incubated overnight in DMEM containing 1% heat-inactivated FBS in the absence or presence of 100 ng/ml pertussis toxin (+PTX) to uncouple Gi signaling. Subgroups of aortas were also incubated ex vivo for 4 hours with 10 μM VPC23019 (+VPC23019), an S1P1 receptor antagonist, immediately before performing a monocyte adhesion assay directly on the aortic tissue. *Significantly higher than saline control, P < 0.0001; ** significantly lower than TNF, P < 0.0001; significantly higher than saline control, P < 0.05 by ANOVA. Mean ± SE of 5 mice per group. B, C57BL/6J mice were injected intravenously with saline or 0.2% FAFBSA (saline), or with 500 ng murine TNFα for 2 hours (+TNF), or with 500 ng murine TNFα plus 5 mg/kg SEW2871, a S1P1 agonist for 2 hours (+TNF + SEW2871). A subgroup of aortas was also incubated with 100 nM 12SHETE (+12SHETE) as a lipid control. *Significantly higher than saline control, P < 0.0001; ** significantly lower than TNF, P < 0.0001; significantly higher than saline control, P < 0.05 by ANOVA. Mean ± SE of 5 mice per group. C, Representative images from aorta of C57BL/6J mice injected IV with 500 ng recombinant murine TNFα (left) or with 500 ng recombinant murine TNFα plus 5 mg/kg SEW2871 for 2 hours (right). After injections, aortas were harvested and immediately incubated with fluorescently-labeled monocytes at 37°C for 30 minutes. Images show one representative aorta per experimental group. Fluorescent monocytes can be seen adhering to aortic endothelium. Note the reduction in the number of monocytes bound to the aorta in which the mouse has received an IV injection of the S1P1 agonist SEW2871 (right).
S1P does not modify expression of any of these adhesion molecules significantly. Levels of S1P did not alter the TNF-α-mediated increase in adhesion molecule expression on endothelium, and only modestly impacts secretion of KC.

**Identification of S1P1 as an Antiinflammatory Receptor Pathway in Endothelium**

Mouse aortic ECs express mRNA for only S1P1, S1P2, and S1P3 (Figure II, available online at http://atvb.ahajournals.org). We have found expression of only S1P1, S1P2, and S1P3 in human ECs (data not shown). We transfected human umbilical vein ECs because of their ease of transfection with siRNA to target S1P1 and S1P3; transfection rates approached 95%. S1P1-targeted siRNA, but not S1P3 siRNA, significantly reduced S1P action by ~70% (Figure 3). A scrambled siRNA control had no effect. We confirmed these data using primary human monocytes (data not shown).

Expression of target S1P1 mRNA was decreased by 70% and target S1P3 mRNA by 55% (Figure III, available online at http://atvb.ahajournals.org). We have found expression of only S1P1, S1P2, and S1P3 in human ECs (data not shown). We transfected human umbilical vein ECs because of their ease of transfection with siRNA to target S1P1 and S1P3; transfection rates approached 95%. S1P1-targeted siRNA, but not S1P3 siRNA, significantly reduced S1P action by ~70% (Figure 3). A scrambled siRNA control had no effect. We confirmed these data using primary human monocytes (data not shown).

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human umbilical vein ECs with 100 nM S1P did not reduce TNF-α–mediated activation of NFκB in endothelium (data not shown). However, S1P prevented TNF-α–mediated activation of a c-jun-luciferase reporter plasmid (Figure 4). S1P significantly increased phosphorylation of Akt (Figure 5). Akt phosphorylation by S1P upregulates eNOS activity.23 Our results suggest that the protective action of S1P on endothelium is caused, at least in part, by upregulation of Akt phosphorylation and decreased activation of c-jun.

Discussion

Monocyte–endothelial interactions are initiating events in vascular inflammation. In the current study, we show that S1P prevents TNF-α–mediated monocyte adhesion to endothelium. This is the first report to our knowledge that indicates that S1P binding to the S1P1 receptor on endothelium activates a potent antiinflammatory signaling cascade and prevents initiation of inflammation in aorta in response to TNF-α. In that TNF-α is a primary cytokine produced during inflammation, the action of the S1P1 receptor in the vasculature has broad therapeutic implications.

S1P receptor agonists modulate cell migration, lymphocyte homing, and vascular permeability. Recent reports have identified that FTY720, a nonselective S1P receptor agonist, modulates lymphocyte homing,24–26 FTY720 is a sphingosine analog that is rapidly phosphorylated in vivo to yield an active metabolite that is an agonist at 4 of 5 S1P receptors. FTY720 regulates vascular permeability, thereby protecting against acute lung injury in mice.27 However, SEW2871 is the first known specific agonist of the S1P1 receptor. SEW2871, like S1P, has been shown to modulate lymphocyte homing and influence cell migration.11 Our data support a new role for S1P1 agonists in modulating monocyte–EC interactions in inflammation. Possibly, S1P1 agonists exert antiinflammatory effects in monocytes as well as in endothelium. The role of S1P1 agonists, including SEW2871 and FTY720, in modulating monocyte trafficking to tissues is also an important concept that is examined.

In the current study, we did not inject mice in vivo with a bolus amount of S1P. Bolus injection of S1P is toxic to mice, probably because of cardiovascular effects mediated through S1P3.17 Although slow infusion of S1P may be possible,28 we did not find this method relevant for studying acute responses to TNF-α in vivo. We were unable to confirm downregulation of S1P1 receptor protein in response to siRNA because of lack of specific antibodies to S1P1 and S1P3. However, we did observe a 70% knockdown of S1P1 receptor mRNA, so we predict that the S1P1 receptor protein levels are also significantly reduced.

Our data point to S1P1 as being the primary endothelial S1P receptor that prevents monocyte adhesion to aorta. We made this conclusion based on results from a number of different assays using pharmacological reagents as well as molecular siRNA. In Figure 3, we found a small, yet significant, effect of the S1P3 siRNA on TNF-α–mediated monocyte adhesion. We did not have specific S1P3 pharmacological reagents for our studies. Although the bulk of our data point to S1P1 as the primary S1P receptor preventing TNF-α–mediated monocyte–EC interactions, we cannot absolutely rule out some contribution of S1P3. This is especially important in light of the fact that S1P3, like S1P1,23 has recently been shown to regulate eNOS activity.13 We examine the role of S1P3 in regulating these early inflammatory processes.

The mechanisms by which S1P exerts its antiinflammatory effect are still unclear. We examined expression of adhesion molecules on endothelium in response to S1P. TNF-α upregulates expression of VCAM-1 and ICAM-1 on the endothelial surface.29,30 S1P at 100 nM did not reduce the TNF-α–mediated increase in endothelial VCAM-1 or ICAM-1 expression. Previous studies21,31 reported that S1P induced endothelial VCAM-1 and E-selectin expression. These investigators used very high concentrations of S1P (1 to 20 μmol/L). We recapitulated this finding, but only when S1P was added to ECs at concentrations >5 μmol/L (data not shown). In the bloodstream, physiological levels of free S1P are in the nanomolar range6,7 and the reported Kd values for S1P binding to the S1P1 receptor are 1 to 10 nanomolars.
Thus, it is conceivable that very high concentrations of S1P act on ECs through some other signaling pathway. Xia et al reported that S1P at concentrations >1 μmol/L activated NFκB.31 These investigators also reported that TNF-α activated sphingosine kinase in endothelium (the enzyme that generates S1P from sphingosine), causing increased production of S1P that leads to erk, c-jun, and NFκB activation. We also observed NFκB activation in endothelium by S1P concentrations of 1 μmol/L or greater (data not shown). However, at concentrations of S1P <500 nM, we found no activation of NFκB, and a decrease in c-jun activation in response to TNF-α stimulation of endothelium. Again, because we are able to recapitulate these investigators’ findings when using micromolar concentrations of S1P, we believe that the concentration of S1P in circulation is quite important for preventing inflammatory processes. Thus, the antiinflammatory action of S1P is through nanomolar concentrations of S1P activating primarily S1P1 on endothelium. Xia et al reported that pertussis toxin (to uncouple Gi signaling of GPCRs as shown in Figure 1) did not inhibit the effect of S1P on adhesion molecule expression, suggesting that S1P at higher concentrations acts intracellularly on some non-S1P receptor pathway.31 However, we did not measure sphingosine kinase activation in the current study, so we do not know if injection of TNF-α in vivo increased sphingosine kinase activity. However, in light of our data supporting a decrease in monocyte adhesion in response to S1P, this would seem unlikely.

A second mechanism of action of S1P on endothelium could be to reduce endothelial production of proinflammatory chemokines, including IL-8 and MCP-1. We found that TNF-α increased endothelial production of both JE/MCP-1 and KC. S1P modestly reduced protein expression of KC (by ∼10%), but not JE/MCP-1. We did not examine chemokine expression at later time points. IL-8/KC has been shown to be regulated by NFκB, p38 MAP kinase, and by Jun kinase pathways through activation of the transcription factor AP-1. We have previously reported endothelial IL-8 activation by p38 MAP kinase and AP-1 activation in diabetes.20 Activation of these signaling pathways in endothelium may be the result of increased oxidative stress in response to TNF-α. We did not examine p38 activation by TNF-α and S1P in the current study in that inhibition of p38 MAP kinase by 5 μmol/L SB203580 had no impact on S1P action in a monocyte adhesion assay (data not shown). However, we did find that S1P reduced activation of Jun kinase (Figure 4). Thus, our data suggest that endothelial KC/IL-8 mRNA levels may be reduced by S1P through inhibition of JNK and AP-1. Studies are ongoing to further investigate this finding.

Garcia et al have recently reported that 500 nM S1P enhanced endothelial barrier function in the pulmonary vasculature.23 This enhanced barrier protection in EC was caused by a tighter rearrangement of the cytoskeleton that was mediated by S1P. It is unclear whether enhanced barrier function in EC prevents monocyte–EC interactions in the vessel wall, although a correlation is quite possible. Sessa et al have shown that S1P regulates Akt and eNOS expression.23 Thus, signaling mechanisms downstream of S1P1 that include activation of PI3 kinase and Akt may be protective against monocyte–endothelial interactions in that reports have suggested that eNOS prevents leukocyte–endothelial interactions and may enhance barrier function.34,35 Additional evidence that points to a role for eNOS in S1P action in endothelium is a report by Natarajan et al that suggested that nitric oxide production by the endothelium prevented Jun kinase-mediated activation of AP-1.22 Michel et al have also reported that S1P caused vasodilation in rat arteries through rapid phosphorylation and activation of eNOS.12 Studies are underway in the laboratory to examine the possible role of eNOS in the antiinflammatory action of S1P on monocyte–EC interactions.

In conclusion, we have identified that S1P signaling to the S1P1 receptor on EC protects the vasculature against TNF-α-mediated monocyte–EC interactions. Identification of this potent antiinflammatory action of S1P and understanding its signaling mechanism may be of great therapeutic benefit for a number of inflammatory diseases.

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Figure la

Relative expression mRNA/cyclophilin

- No Treatment
- +TNF
- +TNF+100nMS1P

KC MCP-1

*  **  *

Figure la
Figure Ib

The bar graph shows the levels of KC and MCP-1 in different treatment conditions. The horizontal axis represents the treatment groups, which include No treatment, +TNF, +S1P, and +TNF+100nM1P. The vertical axis represents the concentration of KC and MCP-1 in pg/mg protein.

Significant differences are indicated by asterisks: *

Legend:
- No treatment
- +TNF
- +S1P
- +TNF+100nM1P

The graph indicates that +TNF and +S1P treatments significantly increase the levels of KC and MCP-1 compared to the No treatment group. The +TNF+100nM1P treatment also shows a trend in increasing KC and MCP-1 levels.

This demonstrates the effects of these treatments on KC and MCP-1 production in the experimental model.
Figure II
Online Methods

Reagents. Recombinant human and mouse TNFα were obtained from R&D Systems. S1P was obtained from Cayman Chemicals. VPC23019 was synthesized in the laboratory of Dr Timothy Macdonald, University of Virginia. SEW2871 was obtained from Maybridge (Cornwall, UK). Human umbilical vein EC (HUVEC) were kindly provided by Dr Brett Blackman, University of Virginia. Pertussis toxin was kindly provided by Dr Erik Hewlett, University of Virginia. SiPORTamine transfection reagent was obtained from Ambion. Antibodies for flow cytometry were purchased from BD Biosciences and included mouse VCAM-1 (#555647), ICAM-1 (#555511), and E-selectin (#551145). Antibodies recognizing phospho-Akt (ser 473; #4058P) total Akt(clone 5G3; #2966) were obtained from Cell Signaling. Mouse KC ELISA kits were purchased from Pierce Endogen, and mouse JE/MCP-1 ELISA kits were purchased from R&D Systems.

Resuspension of S1P and SEW2871. S1P was dissolved in 95% DMSO/5% 1N HCl in H2O at a concentration of 20 mM S1P. This 20 mM solution was further diluted 20:1 into 3% fatty acid free BSA (FAFBSA) in 1X PBS to yield a final stock concentration of 1mM S1P in 3% FAFBSA/5% acidified DMSO. Further dilutions of the 1 mM S1P stock solution for cell culture use were diluted into 0.2% FAFBSA/PBS and added directly to cells.

SEW2871 was dissolved in PBS with 3% FAFBSA/5% acidified DMSO to yield a final concentration of 1 mM. Further dilutions of this 1 mM SEW2871 stock solution were diluted into 3% FAFBSA/sterile saline and injected into mice, or diluted into 3% FAFBSA/PBS and added directly to cells.
Mouse Aorta Isolation and Monocyte Adhesion Assay: Male C57BL/6J mice (designated B6; 8-10 weeks old) were purchased from Jackson Laboratories (stock # 000664) and maintained on rodent chow. All animal studies were performed following approved guidelines of the University of Virginia Animal Care and Use Committee. Mice were injected intravenously with 500 ng recombinant murine TNFα, or with 500 ng TNFα plus 5 mg/kg of the S1P1 agonist SEW2871. At 2h post-injection, aortas were harvested from the mice, and immediately placed into DMEM + 1% heat-inactivated FBS. The aortas were opened up longitudinally, and pinned onto sterile agar. All aortas were incubated overnight in above media at 37°C. A subgroup of aortas were treated overnight with 100ng/ml pertussis toxin in DMEM +1% heat-inactivated FBS. After incubation, aortas were incubated for 30 mins with $1 \times 10^6$ fluorescently-labeled (using Calcein AM) WEHI 78/24 mouse monocytes. Monocytes were labeled with Calcein–AM (Molecular Probes) according to manufacturer’s instructions. After incubation, unbound monocytes were rinsed away, and the number of monocytes firmly bound to aorta was counted in 3 consistent fields using fluorescent microscopy. Data are represented as the mean±SE of 3 areas of aorta. The areas counted were within a grid, and the same locations per aorta were counted. In a subgroup of mice, after TNFα injection and aorta harvest, the aortas were immediately incubated for 4h with 100 nM S1P ex vivo prior to performing a monocyte adhesion assay. Some groups of aortas were incubated for 4h immediately prior to performing a monocyte adhesion assay with 10 µM SEW2871, a S1P1 receptor agonist. As controls for injection, B6 mice were
injected with 150 µl saline+0.2% fatty acid-free BSA (FAFBSA) intravenously.

**Cell culture:** Mouse aortic endothelial cells (MAEC) were obtained from isolated aorta from C57BL/6/J mice as previously described\(^1\). MAEC were cultured in Medium 199 containing 15% heat-inactivated FBS, 20µg/ml ECGS, and 90µg/ml heparin. Cells were used at passage 2.

HUVEC were cultured in Medium 199 containing 20% heat-inactivated FBS, 20µg/ml ECGS, and 90µg/ml heparin and used from passage 3 to 6. The experimental use of HUVEC was approved by the University of Virginia Institutional Review Board (IRB), and all procedures were performed in accordance with University IRB guidelines.

**Mouse monocyte adhesion assay:** MAEC were cultured to confluency in 48-well plates. WEHI78/24 monocytes were labeled with Calcein-AM and 35,000/well were added to MAEC monolayers. A monocyte adhesion assay was performed as previously described\(^1,2\).

Mouse recombinant TNF\(\alpha\) (10U/ml), S1P (100 nM), VPC23019 at 10 µM, and SEW2871 at 10 nM, 100 nM and 1µM were added to the MAEC for 4h at 37°C prior to performing a monocyte adhesion assay. Pertussis toxin (100ng/ml) was added to MAEC for 16h prior to incubation with TNF or S1P.

**Flow chamber studies.** MAEC were cultured to confluency in a parallel plate flow chamber (Glycotech). MAEC were treated with 10U/ml recombinant murine TNF\(\alpha\) ±100 nM S1P for 4h at 37°C. WEHI 78/24 mouse monocytes (1x10\(^6\) cells/ml) were allowed to flow over the endothelium at a shear stress of 0.75 dynes/cm\(^2\). Data was recorded for 5 minutes using a video recorder as
previously described\textsuperscript{3-5}. The total number of rolling monocytes and the total number of monocytes firmly adherent to endothelium at the end of 5 minute periods were counted. Triplicate plates were performed for each experimental condition.

**Human monocyte adhesion assay:** HUVEC were cultured to confluency in 48-well plates as described above. Human recombinant TNF\(\alpha\) (10U/ml) and/or S1P (100 nM) was added to EC for 4h at 37C. MonoMac 6 cells (MM6), a human monocyte cell line that has been well-characterized\textsuperscript{6,7} were labeled with Calcein-AM and 50,000/well were added to EC monolayers for a monocyte adhesion assay\textsuperscript{8,9}.

**KC/IL-8 and JE/MCP-1 measurements.** Total RNA was isolated from MAEC and cDNA was prepared as described previously\textsuperscript{10-12}. For quantitative measurements of KC and JE/MCP-1 mRNA, 2 \(\mu\)ls of cDNA from each experimental group were utilized. Real-time PCR was performed as we have described previously\textsuperscript{10-12}, and mouse cyclophilin was used as a housekeeping control for normalization purposes. Nanograms of KC mRNA were calculated using the relative expression method. Mouse KC and Mouse JE/MCP-1 protein secretion was quantified using ELISA in culture supernatants of MAEC treated for 4h with TNF±100 nM S1P. For mouse KC, 50\(\mu\)l/well of a 1:20 dilution of culture supernatant was analyzed, and for mouse MCP-1 200\(\mu\)l/well of a 1:2 dilution of culture supernatant was analyzed. Values were determined based upon a standard curve for each chemokine, and total quantities of chemokine were normalized to mg of total cell protein to control for possible cell number
differences in each plate.

**Flow cytometry.** MAEC at passage 3 were collected in PBS by gentle scraping using a cell scraper. 150,000 cells per sample were analyzed for each antibody. Cells were incubated for 30 min at 4°C with FITC-labeled primary antibodies for mouse adhesion molecules (FITC anti-mouse ICAM-1, FITC anti-mouse VCAM-1, FITC anti-mouse E-selectin) as described previously². Samples were analyzed at the University of Virginia Flow Cytometry Core using a Becton Dickinson FACSCalibur™ flow cytometer. FITC fluorescence was collected in the FL1 channel through a 530/30 nm bandpass filter. Analyses were performed using FL1 channel histograms from which the mean fluorescence intensity (MFI) channel was calculated for each sample.

**Immunoblotting for Akt.** HUVEC were harvested in 1X cell lysis buffer (Cell Signaling, Inc) in the presence of a protease inhibitor cocktail (Sigma). 50µg of total HUVEC protein was analyzed by 4-12% SDS-PAGE in MOPS running buffer and transferred to nitrocellulose. Pierce Blocker BLOTTO in TBS was used as a blocking agent. Membranes were probed with a 1:2000 dilution of phospho-Akt (ser 473) antibody. Phosphoproteins were detected using a 1:2000 dilution of anti-rabbit IgG-HRP and chemiluminescence. Bands were normalized to total Akt protein, and quantitated using densitometry.

**Measurement of c-Jun transcriptional activity.** HUVEC were transfected using the Amaxa Nucleofector II and Basic Endothelial Nucleofector Kit according to the manufacturer’s protocol. The M3 setting was determined to provide the highest transfection efficiency (>70%) with the least amount of cell
c-Jun activity in HUVEC was assayed using the PathDetect Signal Transduction Pathway trans-reporting System (Stratagene) following the manufacturer’s protocols. At 48h post-transfection, cells were treated for 2h with 10U/ml TNFα ± 100nM S1P for 30 mins. Luciferase activity was measured using a Berthold Syrius Luminometer (Fisher Scientific).

Transfections with siRNA. S1P1, S1P3 and scrambled control siRNA were designed using the Ambion siRNA Design Tool™ program. HUVEC were plated in a 6-well dish at 40% confluency, and were transfected with 10 nM of siRNA using 5 µl of SiPORTamine reagent/well. Transfection conditions were optimized following the manufacturer’s protocol. Total RNA was extracted 48 hrs after the transfection. Reverse transcription of 2 µg of total RNA was performed as described previously10-12. For quantitative measurements of S1P receptor mRNA, 2 µls of cDNA from each experimental group were utilized. Quantitative PCR conditions were 95ºC 5mins, followed by 40 cycles of 94ºC 30 secs, 60ºC 30 secs, 72ºC 45 secs, followed by one cycle of 95ºC for 1 min. Primers to detect knockdown of target mRNAs were: S1P1 forward: 5'-tatcatgctcggcattaca-3’ and S1P1 reverse: 5’- gaacaccaccgaggtcagtt-3’; S1P3 forward: 5'- ggcatcgcttacaaggtcaa-3’ and S1P3 reverse: 5’- gaacatactgccctcctga-3’. Samples were normalized to human tubulin as a housekeeping mRNA control.

Statistical Analyses: Data for all experiments were analyzed by ANOVA using the Statview 6.0 software program. Comparisons between groups were performed using analysis of variance (ANOVA) methods. Data are graphically
represented as mean ± SE, in which each mean consists of six experiments performed in quadruplicate (unless noted otherwise in the figure legends) using cells isolated from 6 mice per group. Aorta studies ex vivo were performed using 4 mice per group. HUVEC studies were performed in quadruplicate using 4 individual sets of cell preparations. Comparisons between groups and tests of interactions were made assuming a two-factor analysis with the interaction term testing each main effect with the residual error testing the interaction. All comparisons were made using Fisher's LSD procedure, so that multiple comparisons were made at the 0.05 level only if the overall F-test from the ANOVA was significant at p<0.05.
Online Figure Legends

**Figure I. Expression of chemokines modulated by TNFα and S1P.** Aortic MAEC at passage 2 harvested from C57BL/6J mice were treated for 4h with 10U/ml murine TNFα (+TNF), or TNFα plus 100 nM S1P (+TNF+S1P). Panel A. Chemokine mRNA levels. Total RNA was isolated from cells and cDNA prepared as described in Materials and Methods. Real-time quantitative Taqman PCR for mouse KC and JE/MCP-1 was performed as described. *Significantly higher than no treatment, p<0.0001; # significantly lower than TNF, p<0.01; **significantly lower than TNF, p<0.001 by ANOVA. Panel B. Secretion of chemokines from MAEC. Media was collected from MAEC treated as described above, and was analyzed for secretion of KC and JE/MCP-1 by ELISA. Values were normalized to mg total cell protein. *Significantly higher than no treatment, p<0.0001; # significantly lower than TNF, p<0.05 by ANOVA.

**Figure II. S1P receptor expression in mouse aortic EC.** RNA was isolated from MAEC and used in RT-PCR to detect S1P receptors. There was no detectable expression of S1P4 or S1P5 mRNAs in MAEC (data not shown). Lanes 1-3: Each lane shows primary MAEC freshly isolated from an individual C57BL/6J mouse. Lane 4: PCR H2O blank. Expected product sizes: S1P1: 778bp; S1P3: 466bp; S1P2: 425 bp; β-actin: 716 bp.

**Figure III. Specificity of S1P receptor siRNA for target mRNA.** RNA was analyzed by real-time quantitative PCR from HUVEC that were transfected with S1P1 siRNA (+S1P1siRNA), S1P3 siRNA (+S1P3siRNA), or scrambled, control siRNA (+CTRsRNA). Untransfected cells were maintained in media as a
negative control (No Rx). PCR for specific S1P receptors was performed as described in Methods. Each S1P receptor siRNA (S1P1, left panel and S1P3, right panel) caused a significant, specific reduction in target mRNA.

*Significantly lower than CTRsiRNA or No Rx for S1P1, P<0.001; #significantly lower than CTRsiRNA or No Rx for S1P3, p<0.01 by ANOVA.


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