LPS and sphingosine 1-phosphate cooperate to induce inflammatory molecules and leukocyte adhesion in endothelial cells

Isabel Fernández-Pisonero*¹, Ana I. Dueñas†, Olga Barreiro‡, Olimpio Montero§, Francisco Sánchez-Madrid†, Carmen García-Rodríguez*∞

*Instituto de Biología y Genética Molecular (CSIC-Universidad de Valladolid); †Hospital Clínico Universitario, Valladolid; ‡Servicio de Inmunología, Hospital de la Princesa, Instituto de Investigación Sanitaria de la Princesa and Centro Nacional de Investigaciones Cardiovasculares (CNIC), Madrid; §Centro para el Desarrollo de la Biotecnología, CSIC, Valladolid

¹I.F.P. and A.I.D. contributed equally to this work

Running title: LPS and S1P cooperation on inflammatory molecules

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Address correspondence: Carmen García-Rodríguez, Instituto de Biología y Genética Molecular C/ Sanz y Forés 3, 47003-Valladolid, Spain. E-mail address: cgarcia@ibgm.uva.es. Phone: +34-983-184841; FAX: +34-983-184800

Abbreviations used in this article: HAEC, human aortic endothelial cells; S1P, sphingosine 1-phosphate; TLR, Toll-like receptor.
Abstract

Given that Toll-like receptors (TLRs) and sphingosine-1-phosphate (S1P) are key players in inflammation, we explored the potential interplay between TLRs and S1P in the adhesion/inflammatory pathways in primary human endothelial cells. As determined by Western blot and flow cytometry, cells treated with LPS, a TLR4 ligand, and S1P showed significantly enhanced expression of adhesion molecules such as ICAM-1 and E-selectin as compared with the effect of either ligand alone. Cell-type differences on E-selectin up-regulation were observed. In contrast, no cooperation effect on ICAM-1 or E-selectin was observed with a TLR2/TLR1 ligand. Consistent with increase in adhesion molecule expression, endothelial cell treatment with LPS + S1P significantly enhanced adhesion of peripheral blood mononuclear cells under shear stress conditions, as compared with the effect of either ligand alone, and exhibited comparable levels of cell adhesion strength than TNF-α. Moreover, LPS and S1P cooperated to increase the expression of pro-inflammatory molecules like IL-6, cyclooxygenase-2, and prostacyclin, as determined by ELISA and Western blot. The analysis of signaling pathways revealed the synergistic phosphorylation of ERK upon LPS+S1P treatment of HUVEC and HAEC, and cell-type differences on p38 and NF-κB activation. Moreover, pharmacological and siRNA experiments disclosed the involvement of S1P1/3 and NF-κB in the cooperation effect and that cell origin determines the S1P receptors and signaling routes involved. SphK activity induction upon LPS+S1P treatment suggests the S1P/Sphk axis involvement. In summary, LPS and S1P cooperate to increase pro-
inflammatory molecules in endothelial cells and, in turn, to augment leukocyte adhesion, thus exacerbating S1P-mediated pro-adhesive/pro-inflammatory properties.
**Introduction**

Sphingosine 1-phosphate (S1P) is a bioactive lipid mediator that mediates a wide spectrum of cell functions in both intracellular and extracellular compartments, which includes proliferation, migration, differentiation, angiogenesis, and lymphocyte trafficking (1-3). Many of S1P actions are mediated by the activation of receptors, initially known as EDGs (endothelial differentiation gene) and now designated as S1P1-5, which are high affinity receptors coupled to G proteins (αio, αq or α12/13) that trigger the activation of multiple signals leading to specific cell responses (4). In addition, S1P can play an important role as a second messenger during inflammation (2). Accumulating evidence suggests an important role of S1P in the regulation of the immune system and the vascular system (2, 5). The S1P/S1P-receptor signaling axis has been implicated in a variety of pathophysiological conditions and diseases such as atherosclerosis, cancer, diabetes, multiple sclerosis, sepsis etc, and interference of these routes has the potential for the treatment of chronic inflammatory disorders and autoimmune diseases (6-7).

The physiological effect of extracellular S1P in vascular biology is dictated by the S1P receptor subtype distribution and the G-proteins involved in its signaling (5). With regard to its physiopathological role, even though it has been suggested that S1P could explain in part the atheroprotective effects of HDL, both S1P-mediated anti-atherogenic and pro-atherogenic effects have been reported in the vascular system (8-10). In endothelial cells, S1P promotes the expression of adhesion molecules associated to angiogenesis and atherosclerosis processes...
as well as inflammation-related genes, although it also promotes anti-atherogenic effects (8-11).

Toll-like receptors (TLR) are sensors of microbial components that play an important role on innate immunity (12) and the pathogenesis of several inflammatory diseases such as systemic lupus erythematosus, sepsis, atherosclerosis (13-14). Genetic and experimental evidences link TLR4, the first receptor identified in humans and a sensor of LPS, an outer component of gram-negative bacteria, to atherogenesis (14). In endothelial cells, TLR activation has been reported to promote lipid uptake and adhesion of leukocytes in the atherosclerotic lesions (14).

Interplay between TLRs and G-protein coupled receptor is known to modulate immune responses (15-16). Given the role of TLR activation in promoting leukocyte accumulation within atherosclerotic lesions (14), and in light of previous studies demonstrating the role of S1P in the induction of adhesion molecules (17-18), we sought to investigate the potential interplay of TLR and S1P receptors in the adhesion and inflammatory pathways in human endothelial cells from venous and arterial origin.
Materials and methods

Reagents

HUVEC, HAEC (human aortic endothelial cells), endothelial cell growth medium-2 (EGM®-2, endothelial medium supplemented with growth factors and cytokines), fetal bovine serum (FBS), nucleofector kit for HAEC and antibiotics were purchased from Clonetics-Lonza (Walkersville, MD). M199, TRIzol and lipofectamine 2000 were from Invitrogen (Carlsbad, CA). Biocoll gradients were from Biochrom KG (Berlin, Germany). Pam3CSK4 was from InvivoGen (San Diego, CA). LPS from E. coli type 0111:B4 (#L2630), S1P (#S9666), SP600125, anti-human β-tubulin antibody (#T7816), and pertussis toxin (PTX) were purchased from Sigma (St. Louis, MO). S1P was dissolved in methanol, following manufacturer instructions; LPS and Pam3CSK4 were dissolved in endotoxin-free water. Anti-human ICAM-1 (#sc-1511), and cyclooxygenase (COX)-2 (#sc-1745) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). PE-conjugated anti-human ICAM-1 antibody was from Diaclone (Beçanson, France). PE-conjugated anti human E-selectin antibody was from eBioscience (San Diego, CA). Human TNFα was from Preprotech (Rocky Hill, NJ). Parallel plate flow chambers were from Willco-Dish (Amsterdam, The Netherlands). ECL, IL-6 immunoassay kit, and [γ-32P]ATP (3000 Ci/mmole) were from Pierce (Rockford, IL). 6-keto-PGF1α immunoassay kit was from GE Healthcare (Buckinghamshire, United Kingdom). Human inflammation antibody array III was from RayBiotech (Norcross, GA). Phospho-NF-κB p 65 (ser 536) antibody (#1673031S) was from Cell Signalling (Danvers, MA), anti
ERK1/2 (#V1141) and anti-ACTIVE® MAPK, p38 and JNK polyclonal antibodies were from Promega (Madison, WI). The permeable inhibitor peptide, NF-κB SN50, ALLN, and SB203580 were from Calbiochem (Darmstadt, Germany). VPC 23019 and S1P were purchased from Avanti Polar Lipids (Alabaster, AL). Suramin was from Biomol (Santa Fe, NM). PD98059 and JTE-013 were from Tocris (Bristol, UK). Transwell® plates were purchased from Corning Inc. (Corning, NY). siRNA duplexes were from Ambion Inc (Austin, TX). A human ICAM-1 monoclonal antibody Hu5/3 antibody was used for immunofluorescence studies (19). Recombinant sphingosine kinase (SphK)-1 and D-erythro sphingosine were from Echelon Biosciences Inc. (Salt Lake City, UT).

**Culture of endothelial cells and mononuclear cell isolation**

HUVEC were cultured in M199 supplemented with antibiotics and 20% FBS. HAEC were cultured in EGM®-2 supplemented with antibiotics, and 2% FBS. Cells between passages 3 and 7 were used in the experiments. PBMCs were isolated from blood collected from healthy volunteers at the Princesa Hospital (Madrid, Spain) by using Biocoll gradients. The investigation, approved by the CSIC ethics review board, conforms to the principles outlined in the Declaration of Helsinki.

**Real-time RT-PCR analysis**

RNA was isolated by the TRIzol method following manufacturer’s protocol. First-strand cDNA was synthesized from total RNA by the reverse transcription reaction, and later amplified by PCR. Primer sequences for human S1P receptors and TLRs were as described
cDNA was amplified in a PTC-200 apparatus equipped with a Chromo4 detector (BioRad) using SYBR Green I mix containing HotStart polymerase (ABgene). β-actin was used as a housekeeping gene to assess the relative abundance of mRNA.

**Immunodetection of ICAM-1, COX-2, pNF-κB, and MAP kinases**

Cells were stimulated with TLR ligands (0.1-5 μg/mL LPS, or 100-300 ng/mL Pam3CSK4) in the presence or not of S1P (0.1-5 μM), and cell lysates analyzed by Western blot as described earlier (20). Expression of pro-inflammatory molecules was detected with antibodies against human COX-2 and ICAM-1. NF-κB activation was measured using an anti-phospho-NF-κB p65 antibody. MAP kinase activation was evaluated using phosphospecific antibodies for the phosphorylated forms of ERK, JNK, and p38 MAPK. Equal loading across the gel was confirmed using anti-β-tubulin or ERK1/2 antibodies. Bands were scanned with a GS-800 calibrated imaging densitometer (Bio-Rad), and acquisition was performed with Quantity One analysis software (Bio-Rad).

**Flow cytometry**

Adhesion molecule expression was analyzed using cells stained with either PE-conjugated anti-ICAM-1, or PE-conjugated anti-E-selectin antibodies, or the corresponding isotype-specific control antibody. Flow cytometry analysis was performed in a Beckman Coulter device (Epics XL-MCL). Data was analyzed using WinMDI software.

**Analysis of endothelial cell-leukocyte adhesion**
Cell-cell adhesion was evaluated by using a parallel-plate flow chamber analysis, as described (19, 22). HAEC monolayers were incubated overnight with culture media containing either vehicle or the corresponding ligands, and later placed in the chamber, and perfused with assay buffer (HBSS with 2% FBS) up to 5 min. Next, PBMCs (2x10⁶/mL) were perfused and allowed to bind to the endothelial monolayer under static conditions for 2 min and then increasing shear stress was applied, starting at 0.5 dynes/cm² and increasing up to 15 dynes/cm² at 1-min intervals. The number of cells attached was quantified throughout eight different fields using Image J software. Images were obtained with a confocal laser-scanning unit (TCS-SP5; Leica) coupled to a microscope (DMI6000; Leica).

Immunofluorescence

Samples removed from the flow chamber were stained with mouse anti-human ICAM-1 and Alexa Fluor 488-labeled goat anti-mouse antibodies. Images were obtained with a fluorescence microscope coupled to a digital camera (DXM1200C Nikon Eclipse 90i), and fluorescence intensity was estimated using Image J software.

Cell migration assay

HAEC (10⁵/well) were seeded on a transwell insert (8 μm-polycarbonate) and the indicated ligands were placed on the lower chamber. Cells were allowed to migrate for 4 h at 37 ºC. Migrated cells were fixed with 3% p-formaldehyde and stained with 0.1 % crystal violet. Images were obtained with a microscope Nikon eclipse TS-100 coupled to a digital camera (Nikon), and cell counting was performed using Image J software.
Antibody arrays and ELISA

Cells were stimulated with the corresponding ligand for 8 h at 37 ºC in M199 with 2% FBS. Supernatants were incubated with a human inflammatory III array to analyze multiple cytokine expression, as described (23), and later used to quantify IL-6 and 6-keto- PGF$_{1\alpha}$ secretion by ELISA following the manufacturer’s protocol.

siRNA experiments

Cells were transfected with lipofectamine 2000 (HUVEC) or by nucleofection (HAEC) following the manufacturer’s protocol. Validated siRNA duplexes specific for S1P$_1$ and S1P$_3$ were as described (20). Quantitative PCR was performed to confirm down-loading of human S1P receptors as described (20). Transfected cells were activated and ICAM-1 and COX-2 were analyzed by Western blot.

S1P quantification by liquid chromatography–tandem mass spectrometry analysis

HAEC were stimulated for 75 min at 37 ºC in M199 with 2% FBS, and washed with saline solution. Lipids were extracted using a modified Bligh and Dyer method with the use of 0.1 N HCl for phase separation, as described. (24) The extracted lipids were resuspended in 100 µL of methanol:water (95:5, v/v) and kept at -80 ºC until analysis. Ultra-performance liquid chromatography interfaced to a time-of-flight mass spectrometry (UPLC-QToF-MS) was performed as described (24) with slight modifications, using an Acquity UPLC System and SYNAT HDMS G2 (Waters, Milford, USA). A two solvent gradient elution was used for
compound separation using an Acquity BEH C18 column (1.7 μm x 2.1mm x 50 mm, temperature 30 ºC), as follows: (i) 0-1 min: 100% A + 0% B; (ii) 1.0-4.5 min: 0% A + 100% B; (iii) 4.5-6.0 min: 0% A + 100% B (isocratic); and (iv) 6.0-8.0 min: 100% A + 0% B. Solvent A consisted of methanol:water:formic acid (50:50:0.5, v/v/v) and 5 mM ammonium formate, and solvent B contained methanol:acetonitrile:formic acid (59:40:0.5, v/v/v) and 5 mM ammonium formate. The flow rate used was 0.5 mL min⁻¹, and the injection volume was 7.5 μL. Compounds were detected as positive ions using a MS² method with low (full scan) and high (fragmentation at 20-30 V) energy functions. MS parameters were: capillary, 0.9 V; sample cone, 18 V; source temperature, 90 ºC; desolvation temperature, 450 ºC; cone gas, 20 L h⁻¹; desolvation gas, 900 L h⁻¹. Quantification was done according to the chromatographic peak area of the peak obtained in the extracted ion chromatogram (EIC) for the m/z 264.269 fragment in the high-energy function. S1P (exact mass 379.25 Da) from Avanti Polar was used to draw an external standard curve for quantification.

_Sphk activity assay_

The Sphk activity was assessed in whole-cell extracts by determining the formation of S1P⁻³²P from [γ⁻³²P]ATP and D-erythro-sphingosine using a protocol described for endothelial cells (25). Samples (10-20 μg) were incubated with 200 μM ATP and 2 μCi of [γ⁻³²P]ATP for 30 min at 37ºC in the Sphk assay buffer (20 mM Tris, pH 7.4, 20% glycerol, 1 mM β-mercaptoethanol, 1 mM EDTA, 1 mM Na₃VO₄, 15 mM NaF, and 0.5 mM 4-deoxypyridoxine). Lipid extraction and liquid scintillation counting was performed as described (25).
Statistical analysis

Results are expressed as mean ± SEM. Data were either analyzed by one-way Anova test or by unpaired t’ test using GraphPad Prism version 4 (GraphPad Prism Software, San Diego, CA). Differences were considered statistically significant for a p < 0.05.
Results

LPS, but not a TLR2/TLR1 ligand, cooperates with S1P to induce surface expression of adhesion molecules

Our aim was to investigate the potential interplay between S1P and TLR ligands on the regulation of the expression and function of adhesion molecules in endothelial cells. First, we evaluated S1P receptor and TLR expression in primary endothelial cells from venous origin, HUVEC, and from arterial origin, HAEC. S1P1 and S1P3 subtype receptors were expressed in both cell types, being S1P1 the most abundant and significantly higher expressed in HAEC than HUVEC (Fig. 1A). As for TLRs, TLR4 was the most abundant receptor, followed by TLR1 and TLR3 (Fig. 1B). To evaluate the effect on adhesion molecules, cells were activated with the vehicle, S1P, LPS, or a combination of S1P and the TLR4 ligand, and cell lysates were later analyzed by Western blot. Densitometry analysis of experiments revealed that ICAM-1 expression was significantly increased following LPS + S1P stimulation in HUVEC as compared with cells treated with either ligand alone (Fig. 1C), and the cooperation effect was observed at 8h after treatment, and lasted at least for 12 h (Fig. 1C). The effect was observed in the range 1-5 μg/mL of LPS (Supplemental Fig. 1A), and 1μg/mL LPS was used in the rest of the study. In HAEC, similar results were obtained (Fig. 1D).

When surface-expression of adhesion molecules was evaluated by flow cytometry, we observed that S1P and LPS cooperated to induce ICAM-1 expression on the cell surface of HUVEC (Fig. 2A) as well as of HAEC (Fig. 2B), in agreement with biochemical data from Fig. 1. The cooperation effect was dose dependent, and it was observed even at low doses of S1P of 100 nM (Fig. 2A-B). On the other hand, the TLR2/TLR1 ligand Pam3CSK4 induced a
small, but statistically significant increase of ICAM-1 expression, consistent with the relatively lower levels of TLR2/TLR1 mRNA in endothelial cells (Fig. 1B), although no additive effect with S1P on ICAM-1 expression in HUVEC (Fig. 2C) and HAEC (Fig. 2D and supplemental Fig 1B) was observed. Interestingly, S1P and LPS did not cooperate to induce surface expression of E-selectin in HUVEC (Fig. 2E and supplemental Fig. 1C), but cooperation was observed in HAEC (Fig. 2F and supplemental Fig. 1C). In contrast, Pam3CSK4 did not show any cooperative effect with S1P even at higher doses neither in HUVEC (Fig 2G) nor in HAEC (Fig 2H). Together, data indicate that LPS and S1P cooperate to induce surface expression of adhesion molecules.

**LPS and S1P cooperate to increase endothelial adhesion to mononuclear cells but not to promote cell migration**

Next, we explored the functional consequences of the up-regulation of adhesion molecules in endothelial cells by analyzing leukocyte-endothelial adhesion under physiological flow conditions. For that purpose, HAEC monolayers were incubated overnight with either vehicle, 1 μg/mL LPS, 1 μM S1P, or a combination of ligands, and 5 ng/mL TNF-α was used as a positive control. Human PBMCs were perfused and allowed to bind to treated-endothelial cells. Remarkably, the number of PBMCs that remained adhered to the endothelial monolayer was significantly higher when endothelial cells were treated with LPS + S1P, as compared to cells treated with either ligand alone (Fig. 3A). Moreover, PBMCs adhered to HAECs monolayers treated with LPS + S1P were highly resistant to laminar flow as compared with cells treated with either ligand alone, and many mononuclear cells still remained attached at
physiologically relevant shear conditions (Fig. 3B), indicating that the LPS and S1P cooperate to increase the strength of PBMC adhesion to endothelial cells. Furthermore, LPS + S1P treatment exhibited comparable levels of cell adhesion strength than TNF-α (Fig. 3B). Next, endothelial cell monolayers with adherent PBMCs derived out of flow analysis were used to confirm that the cell adhesion increment correlated with a parallel up-regulation of ICAM-1 expression. Consistent with the increase in the number of attached PBMCs and the adhesion strength observed in Fig. 3A-B, cells treated with LPS + S1P expressed higher levels of ICAM-1 (17,281 ± 4,266 a.u.) than cells treated with either LPS (7,994 ± 1,048 a.u.) or S1P (1,241 ± 387 a.u.) (Fig. 3C). On the other hand, LPS and S1P showed no cooperative effect on other cell functions, as LPS inhibited S1P-mediated chemotaxis of endothelial cells (Fig. 3D-E). The inhibiting effect of LPS on S1P-induced migration cannot be explained by an LPS-induced down-regulation of S1P1, since LPS showed no effect on S1P1 receptor mRNA levels in real-time RT-PCR experiments (Resting: 6.64 ± 0.11 arbitrary units (a.u.); LPS treatment for 2h: 6.9 ± 0.12, a.u.; LPS 4h: 6.38 ± 0.09 a.u.). On the other hand, S1P did not affect TLR4 levels (Resting: 2.33 ± 0.18 a.u.; S1P 2h: 1.91 ± 0.66 a.u.; S1P 4h: 1.89 ± 0.47 a.u.). Together, these results demonstrate that LPS and S1P strongly cooperate to induce leukocyte-endothelial cell adhesion by significantly increasing cell adhesion strength and the number of attached leukocytes.

*LPS cooperates with S1P in the up-regulation of pro-inflammatory mediators such as IL-6, COX-2 and prostacyclin*
Next, we explored whether LPS and S1P cooperate to induce the expression of pro-inflammatory cytokines using a human inflammation antibody array. Cell treatment with LPS or S1P for 8 h induced the expression of IL-6, IL-8, and ICAM-1, while LPS + S1P further increased ICAM-1 expression, consistent with data from Fig. 1-2, and IL-6 secretion, as observed with a human inflammation antibody array (Fig. 4A). TNF-α was induced by neither LPS nor LPS + S1P (Fig. 4A). Quantification of IL-6 production by ELISA showed that LPS + S1P induced a significantly higher production of IL-6 each ligand alone in HAEC (Fig. 4B), demonstrating a cooperation of LPS and S1P on the expression of this pro-inflammatory cytokine.

Since the pro-inflammatory enzyme COX-2 and its products play an important role in vascular inflammatory responses, we explored the effect of LPS and S1P on COX-2 expression by Western blot. Exposure of HUVEC to either LPS or S1P induced COX-2 expression (Fig. 4C), consistent with previous reports (26-27), whereas cell treatment with LPS + S1P induced a further increase (Fig. 4C). Similar results were observed in HAEC, and the cooperative effect was observed as early as 4h after treatment (Fig. 4D). Next, we tested the effect on the induction of prostacyclin, a major product of COX-2 activity in endothelial cells, by analyzing 6-keto-PGF$_{1\alpha}$ secretion. Consistent with a cooperation effect on COX-2 expression, the levels of secreted 6-keto-PGF$_{1\alpha}$ were significantly higher in cells treated with LPS + S1P as compared with the effect of either ligand alone (Fig. 4E-F). Altogether, these results demonstrate that LPS and S1P cooperate to up-regulate the expression of pro-inflammatory molecules.
Different S1P receptor subtypes and signaling cascades are involved in the cooperation effect in HUVEC and HAEC

To elucidate the S1P receptor subtype(s) involved in the cooperative effect, cells were pretreated with the S1P receptor antagonists before stimulation with LPS + S1P for 8h, and cell lysates were analyzed by Western blot. The cooperative effect of LPS + S1P on ICAM-1 induction was sensitive to PTX, that inhibits Gi/o proteins predominantly coupled to S1P1 but also S1P2/3, and to W146, a S1P1 antagonist, but was affected neither by JTE nor by suramin, S1P2 and S1P3 antagonists, respectively (Supplemental Fig. 2A), suggesting the involvement of S1P1 in HUVEC. These results were further supported by S1P1 silencing experiments (Fig. 5A), and by the S1P1-selective agonist SEW2871 (Supplemental Fig. 2B). On the other hand, in HAEC the effect on ICAM-1 induction was PTX-sensitive, and significantly inhibited by VPC23019, a S1P1/3 antagonist, suramin, and W146 (Supplemental Fig. 2A), and by silencing S1P3 and S1P1 (Fig. 5B), thus suggesting the involvement of S1P3 and S1P1. The cooperative effect on IL-6 production was PTX-sensitive, and significantly inhibited by suramin and W146 in HAEC, indicating the involvement of S1P3 and S1P1 (Supplemental Fig. 2C). With regard to COX-2 induction, the cooperative effect was inhibited by W146, suramin and VPC23019 in HUVEC and HAEC (Supplemental Fig. 2D), and by gene silencing (Fig. 5C-D) suggesting the involvement of S1P3 and S1P1, although differences in PTX sensitivity were observed.

Cell treatment with LPS + S1P activates NF-κB and MAPK routes (Fig. 6A-B). Interestingly, in HUVEC and HAEC, LPS + S1P induced the phosphorylation of ERK in a synergistic manner since the effect was higher that the obtained by the sum of the effect of
each ligand alone (Supplementary Fig 3A-B), suggesting that ERK/MAPK pathway might be a cross-road signalling point. Moreover, in HUVEC, p38 might also serves as a point of confluence (Supplementary Fig 3A). Next we tested the effect of different signalling inhibitors on the cooperative effect on ICAM-1. Blockade of the NF-κB route with ALLN, a proteasome inhibitor, significantly inhibited the induction of ICAM-1 expression by LPS + S1P in HUVEC and HAEC (Fig. 6C-D). Moreover, SN50, that prevents NF-κB translocation into the nucleus, partially inhibited ICAM-1 induction (Fig. 6C-D). As shown in Fig. 6C-D, the effect on ICAM-1 induction was statistically significantly reduced with the ERK inhibitor PD98059 in HUVEC, and to a lower extent by the p38 inhibitor SB203580. In HAEC, those inhibitors and the JNK inhibitor SP600125 showed a partial, but statistically significant, inhibitory effect. With regard to COX-2 induction, the cooperative effect was reduced by blockade of the NF-κB route in HUVEC and HAEC (Fig. 6C-D), and was statistically significant inhibited by SB203580 and by SP600125 in HUVEC, and by SB203580 in HAEC (Fig. 6C-D), thus pointing to the involvement of different MAPK.

Since LPS has been described to increase S1P intracellular levels by sphingosine kinase (SphK)-1 (2), the S1P intracellular content was analyzed by mass spectrometry in HAEC. LPS induced a small increase but not statistically significant, of S1P endogenous levels (Fig. 6E and Supplemental Fig. 4). Exogenous S1P showed a marked increased of S1P endogenous levels, and LPS + S1P induced a further increase (Fig. 6E). SphK activity was detected in HAEC in resting conditions, and treatment with either LPS alone or in combination with S1P induced a small increase in the Sphk activity that was statistically significant with the dual stimulation. Together, data demonstrate the involvement of different S1P receptors and
regulators as well as intracellular signaling cascades in the cooperation with LPS to induce pro-inflammatory molecules in endothelial cells from venous and arterial origin.
Discussion

The present study reveals that exposure of endothelial cells to LPS and S1P leads to a cooperative induction of pro-inflammatory molecules that have a strong impact on leukocyte adhesion under physiologic flow conditions. This might have relevance in inflammatory processes and vascular physiopathology. The first demonstration of interplay between TLR4 and S1P receptors has been addressed in the context of leukocyte extravasation, especially with respect to the induction of adhesion molecules.

Our findings show that concomitant stimulation of endothelial cells with LPS, a bona fide TLR4 ligand, and S1P trigger a series of molecular and cellular events that may contribute to inflammatory vascular diseases. LPS + S1P cooperatively increased the expression of adhesion molecules and other inflammatory molecules thus enhancing the pro-inflammatory/pro-adhesive responses known to be promoted by S1P in endothelial cells (17-18). Up-regulated molecules include ICAM-1, a central player in cell adhesion. E-selectin up-regulation suggests that the cooperation between LPS and S1P is relevant not only to adhesion but to endothelial rolling. Moreover, cooperative effect on E-selectin observed in HAEC but not in HUVEC would argue for an important role of the cooperative effect on the arterial bed, and therefore on the plaque formation.

A firm leukocyte-endothelium interaction/adhesion is an important step in the initiation of the inflammatory processes. In this study, we have shown for the first time that exposure of endothelial cells from arterial origin to LPS + S1P cooperatively increased both adhesion molecule expression and adhesion of mononuclear cells as compared with the effect of either ligand alone. Strikingly, at flow rates similar to physiological (2-5 dynes/cm²), mononuclear
cell adhesion to endothelial cells pretreated with LPS + S1P showed a similar resistance to shear stress than cells pretreated with TNF-α, although the number of cells attached was not as high as with TNF-α, what argues for a minimal threshold of adhesion molecules required for a strong cell adhesion. These observations are consistent with earlier reports showing that exposure to S1P increases surface expression of adhesion molecules in endothelial cells (9, 28) and that the S1P/S1P₃ axis promotes leukocyte recruitment in inflammation and atherosclerosis (29). However, our results differ from studies in which S1P prevents TNF-α-mediated monocyte adhesion to aortic endothelium in mice (30), and S1P protects ischemia/reperfusion via S1P₃, by suppressing leukocyte adhesion (31). It has to be pointed out that there are some conflicting data in the literature, since there are evidences for both pro- and anti-atherogenic effects of S1P, discrepancies that might be explained by species and receptor differences, as well as the S1P dose used. In our study, the cooperation with LPS on adhesion molecules was observed at low doses of S1P, even nM, arguing in favor of a S1P receptor-mediated and physiologically relevant effect.

Our data provide a first demonstration of LPS and S1P cooperation to induce the expression of pro-inflammatory molecules like the cytokine IL-6, a member of the acute phase reactant family known to facilitate leukocyte adhesion to the vascular wall, and to have an effect on other cells in the vascular wall, like smooth muscle cells (32). Our results are in accordance with the cooperation of TLR4 and S1P receptors to enhance inflammatory cytokine production in human gingival epithelial cells (33). On the other hand, in a previous study we have observed that TLR2 and S1P₁/₂ receptors interaction resulted in the inhibition of chemokine production in human monocytes-macrophages (20). These apparent discrepancies could be
explained by the differences in the TLR expression in these cells, since TLR2 expression in endothelial cells is low. Moreover, the S1P receptors involved in the cooperative effect with LPS differ according to endothelial cell type origin, arguing for the importance of cellular context and activation status. Therefore, it is conceivable that the interplay between TLR and S1P receptors might be cell and tissue-specific and most likely determined by the subtype of receptors expressed, as suggested by previous studies (5). In addition to cytokine induction, our data demonstrate that activation of human endothelial cells by LPS + S1P induces a cooperative up-regulation of the pro-inflammatory enzyme COX-2, and synthesis of prostacyclin, the main prostanoid synthesized by vascular endothelium that plays an essential role as regulator of vascular homeostasis. Our data are also in accord with those reporting induction of COX-2 expression by coactivation of mouse intestinal myofibroblasts with S1P and IL-1β, the receptor of which belongs to the IL1R/TLR family (34). Moreover, S1P has been implicated in the regulation of the COX-2 gene, whose products mediate vascular inflammatory responses (26).

The mechanism of cooperation shows cell-specificity on the S1P receptors and intracellular pathways involved, which can account for a different impact on the pathophysiology of venous and arterial endothelial cells. S1P₁/₃ are the predominant receptors in endothelial cells, and, interestingly, cell origin appears to determine the receptor subtypes involved in the cooperative effect. The S1P₁ receptor is involved in the interplay with TLR4 to induce adhesion and cytokine expression in HUVEC, consistent with previous data showing S1P₁-mediated adhesion of U937 cells to HUVEC (35). On the other hand, S1P₃, but also S1P₁, are involved in the cooperation effect in endothelial cells from aortic origin. Interestingly, S1P₁/₃
has been shown to be involved in S1P regulation of inflammation-related genes in human endothelial cells (35). Moreover, S1P₃ has been pinpointed as the receptor involved in pro-adhesive S1P effects, while S1P₁ would account for its anti-adhesive properties in *in vitro* studies, suggesting S1P receptor subtype-specificity (36). Furthermore, the role of S1P₃ in atherogenesis has recently been emphasized by a report demonstrating that S1P₃ promotes recruitment of monocyte/macrophages in inflammation and atherosclerosis (29).

The molecular basis of cooperative induction of inflammatory molecules by LPS and S1P seems to be mediated by intersection on the ERK/MAPK and NF-κB routes. In HUVEC and HAEC, LPS + S1P induced the synergistic activation of ERK, arguing for a role of that pathway in the cooperative effect. Cell-type specific effects were observed, since p38 and NF-κB synergistic induction by dual stimulation is observed in HUVEC, while the effect is additive in HAEC. At the level of pro-inflammatory gene induction, our data with pharmacological inhibitors also suggest cell-type specific effects, In HUVEC, ERK is the main MAPK involved in the ICAM-1 cooperative effect, consistent with the involvement of Gi/o-proteins and S1P₁, while JNK and p38 are involved in the effect in HAEC, pointing to the involvement of G₁₂/₁₃. Our data suggest the involvement of NF-κB p65 proteins in the cooperative effect on the expression of ICAM-1 and COX-2, the promoter of which contains NF-κB binding sites (37-38). These results are consistent with an earlier report showing that S1P-mediated pro-adhesive effects in endothelial cells are mediated by NF-κB (9), a master gene regulator of many pro-inflammatory molecules.

LPS and S1P cooperation could be explained by either interplay between TLR4 and S1P receptors, or by an additive effect. The lack of additive effect of a TLR2/TLR1 ligand and S1P
on ICAM-1 expression, and the observation of no cooperation of LPS, but inhibition of S1P-mediated chemotaxis of endothelial cells, would argue for interplay between TLR4 and S1P receptors. In addition, our MAPK activation data support interplay at the ERK level. Furthermore, mass spectrometry data would favor this hypothesis, since LPS + S1P induced a significant increase of S1P endogenous levels as compared to the effect of exogenous S1P that could not be explained by an additive effect of LPS and S1P, thus arguing for interplay between receptors at the S1P/SphK1 signaling level. The Sphk activity, detected in endothelial cells upon LPS+S1P treatment suggests the involvement of the S1P/Sphk axis, in addition to an interplay at the ERK, which has been reported to act upstream of Sphk1 (39). However, the contribution of TNF-α induced endogenous S1P could not be ruled out since LPS-induced TNF-α expression and TNF-α mediated activation of Sphk1/S1P signaling has been shown (2, 11). The lack of TNF-α induction observed in cytokine arrays after 8h of treatment with LPS + S1P may suggest that the previous observations of LPS on TNF-α could be a secondary response that would not explain the short-term responses we report here with LPS+S1P.

Two challenges/signals engaging cell surface receptors seem to be required to activate a strong inflammatory response in endothelial cells. This can occur upon coincidental exposure to molecular patterns derived from an infection and/or endogenous ligands originated by cell damage and necrosis; and to S1P released after endothelial or red blood cells activation, or after platelet aggregation/activation occurring in active cardiovascular disease states both on a chronic and acute basis (40-41). Based on these data we propose that a two-signal paradigm might be required for an exacerbated inflammatory response, what could underlie the pro-inflammatory mechanism leading to disease.
In summary, our data underscore cooperation between LPS and S1P that leads to an increase in leukocyte adhesion and pro-inflammatory responses in endothelial cells, thus intensifying S1P-mediated pro-inflammatory/pro-adhesive properties, which might have some consequences in vascular physiopathology. As a corollary, our data support a two-signal paradigm required for an exacerbated pathological inflammatory response, and the importance of cellular context.
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References


Figure legends

FIGURE 1. LPS cooperates with S1P to induce ICAM-1 expression in endothelial cells. (A-B) Expression of S1P and TLR receptors in HUVEC (white bars) and HAEC (black bars). Total RNA was analyzed by quantitative RT-PCR using primer sequences for human S1P receptors (A) and for human TLR1-9 (B). Data represent mean ± SEM of the relative mRNA levels normalized to results from the housekeeping gene β-actin. (C) HUVEC and (D) HAEC were incubated with either vehicle, or LPS (1 μg/mL), or S1P (1 μM) or a combination of LPS and S1P for the indicated times, and cell lysates were analyzed by Western blot using ICAM-1 and β-tubulin antibodies. Images are representative of at least n = 5 experiments. Densitometry data, expressed as mean average ± SEM, represent the fold induction increase observed after 8 h of activation when compared to the resting conditions. a.u., arbitrary units. *p < 0.05 as compared to resting conditions; #p < 0.05 for LPS + S1P vs. LPS and S1P.

FIGURE 2. LPS, but not a TLR2/TLR1 ligand, cooperates with S1P to up-regulate surface expression of adhesion molecules in endothelial cells. Cells were incubated with either vehicle, or the indicated ligands for 24h (ICAM-1) or 8 h (E-selectin). Flow cytometry analysis was performed using a PE-conjugated anti-human ICAM-1 antibody (A-D), or a PE-conjugated anti-human E-selectin antibody (E-H). Gating on FS and SS was applied. Histograms are representative of at least 3 independent experiments. Graphs correspond to results expressed as the fold induction increase when compared to the average of the median in resting conditions. Error bars correspond to SEM. *p < 0.05 as compared to resting
FIGURE 3. LPS and S1P cooperate to increase monocyte adhesion to endothelial monolayers but not to promote cell migration. (A-B) PBMCs were perfused through HAEC monolayers previously incubated with the indicated ligands (1 μg/mL LPS, or 1 μM S1P, or LPS + S1P), then increasing laminar flow rates were applied and attached PBMCs were counted. Results are expressed as the number of PBMCs adhered to the HAEC monolayer per field (mean average ± SEM) at the initial flow of 0.5 dynes per cm² (A), and as the percentage of remaining adherent PBMCs at the different flow rates referred to the number of PBMCs after wash out at 0.5 dynes/cm² (B). Data are representative of n = 3 experiments run in duplicate. *p < 0.05 as compared to resting conditions; # p < 0.05 for LPS + S1P vs. LPS and S1P. (C) After flow analysis, ICAM-1 expression was analyzed by immunofluorescence in HAEC monolayers a mAb mouse anti-human ICAM-1 followed by a goat-anti-mouse Alexa Fluor 488-labeled as described in “Materials and methods”. (D-E) HAEC were allowed to migrate in the presence of the indicated ligands (0.1 μM S1P, 1 μg/ml LPS) for 4 h. Images correspond to migrated cells stained with crystal violet as described in “Materials and methods”. Graph corresponds to data expressed as the percentage of migrated cells when compared to S1P treatment (100%). Data, mean ± SEM, are representative of n = 6 independent experiments. **p < 0.05 as compared to S1P. Original magnification x 10 for panels C and D.

FIGURE 4. LPS and S1P cooperate to up-regulate IL-6, COX-2, and 6-keto-PGF₁α expression in endothelial cells. (A) HAEC were stimulated with either vehicle, or LPS (1
μg/mL), or S1P (1 μM) or LPS + S1P for 8 h, and supernatants and cell lysates were analyzed for inflammatory cytokine expression as described in Methods. Images are representative of 2 independent experiments. Squares indicate positive controls; arrows, constitutively expressed cytokines; ovals, the cytokines unambiguously induced upon stimulation; diamond, TNF-α. (B) IL-6 secretion was evaluated by ELISA. Data represent fold induction as compared to resting levels, and is expressed as mean average ± SEM of n = 4 experiments in duplicate. (C-D) HUVEC and HAEC were incubated with either vehicle, or LPS (1 μg/mL), or S1P (1 μM) or a combination of LPS and S1P for the indicated times, and cell lysates were analyzed by Western blot using COX-2 and β-tubulin antibodies. Densitometry data are expressed as in Figure 1. (E-F) Supernatants were analyzed by ELISA for the production of 6-keto-PGF<sub>1α</sub> after 8 h of activation. *p < 0.05 for LPS + S1P vs. LPS and S1P. Data and images are representative of at least 3 experiments in duplicate.

**FIGURE 5.** Different S1P receptors are involved in the interplay with TLR4 in HUVEC and HAEC. siRNA experiments to knockdown S1P receptors were performed as indicated in Methods. Transfected cells were activated with either vehicle, LPS, S1P or LPS + S1P for 8h. (A-B) Cell lysates were analyzed by Western blot with anti-ICAM-1 and anti β-tubulin antibodies. (C-D) Cell lysates were analyzed by Western blot with anti-COX-2 and anti β-tubulin antibodies. Images are representative of 3 independent experiments. Graphs include densitometry results expressed as arbitrary units normalized to β-tubulin data. Scramble indicates a siRNA negative control.
FIGURE 6. Different signaling cascades are involved in the interplay of TLR4 and S1P receptors in HUVEC and HAEC. (A-B) HUVEC and HAEC were incubated with either vehicle, or the indicated ligands for the indicated times, and cell lysates were analyzed using antibodies for the phosphorylated forms of p65-NF-κB, and the MAPK ERK, p38 and JNK. Equal loading was confirmed with an anti-ERK antibody. Images are representative of 4 experiments in duplicate. See supplemental Fig.3 for ratiometric analysis (C-D) Cells were pre-treated with the indicated drugs for 1h before activation with LPS + S1P and lysates were analyzed by Western blot with anti-ICAM-1 and COX-2 antibodies. Images are representative of at least 3 experiments in duplicate. PD indicates 50 μM PD98059; L+S, LPS+S1P; SB, 10 μM SB203580; SN50, 50 μg/mL NF-κB SN50; SP, 10 μM SP600125; R, resting. (E) HAEC were activated with the indicated ligands and S1P intracellular levels were quantified by mass spectrometry and normalized to mg of protein. Data, expressed as fold induction of S1P intracellular content (mean ± SEM) relative to the values obtained after exogenous S1P treatment, is representative of n=3 experiments. *p < 0.05. (F-G) Cells were activated as indicated and lysates were used to measure SpHK activity. Results are expressed as cpm/μg prot.h (F), and fold induction as compared to the untreated cells (G). Data is representative of n = 4 experiments in duplicate. SpHK1 indicates a recombinant protein used as a positive control. * p< 0.05 as compared to untreated cells.
FIGURE 1
FIGURE 2

Fold induction ICAM-1
(mean average)

Fold induction E-selectin
(mean average)

Events

PE-ICAM-1

PE-E-selectin

Resting
S1P
LPS
LPS+S1P

Pam+S1P
Pam
S1P

Fold induction ICAM-1
(mean average)

Fold induction E-selectin
(mean average)

Events

PE-ICAM-1

PE-E-selectin

Resting
S1P
LPS
LPS+S1P

Pam+S1P
Pam
S1P

Fold induction ICAM-1
(mean average)

Fold induction E-selectin
(mean average)

Events

PE-ICAM-1

PE-E-selectin

Resting
S1P
LPS
LPS+S1P

Pam+S1P
Pam
S1P

Fold induction ICAM-1
(mean average)

Fold induction E-selectin
(mean average)

Events

PE-ICAM-1

PE-E-selectin

Resting
S1P
LPS
LPS+S1P

Pam+S1P
Pam
S1P

Fold induction ICAM-1
(mean average)

Fold induction E-selectin
(mean average)

Events

PE-ICAM-1

PE-E-selectin

Resting
S1P
LPS
LPS+S1P

Pam+S1P
Pam
S1P

Fold induction ICAM-1
(mean average)

Fold induction E-selectin
(mean average)

Events

PE-ICAM-1

PE-E-selectin

Resting
S1P
LPS
LPS+S1P

Pam+S1P
Pam
S1P

Fold induction ICAM-1
(mean average)

Fold induction E-selectin
(mean average)

Events

PE-ICAM-1

PE-E-selectin

Resting
S1P
LPS
LPS+S1P

Pam+S1P
Pam
S1P

Fold induction ICAM-1
(mean average)

Fold induction E-selectin
(mean average)

Events

PE-ICAM-1

PE-E-selectin

Resting
S1P
LPS
LPS+S1P

Pam+S1P
Pam
S1P

Fold induction ICAM-1
(mean average)

Fold induction E-selectin
(mean average)

Events

PE-ICAM-1

PE-E-selectin

Resting
S1P
LPS
LPS+S1P

Pam+S1P
Pam
S1P

Fold induction ICAM-1
(mean average)

Fold induction E-selectin
(mean average)

Events

PE-ICAM-1

PE-E-selectin

Resting
S1P
LPS
LPS+S1P

Pam+S1P
Pam
S1P

Fold induction ICAM-1
(mean average)

Fold induction E-selectin
(mean average)

Events

PE-ICAM-1

PE-E-selectin

Resting
S1P
LPS
LPS+S1P

Pam+S1P
Pam
S1P

Fold induction ICAM-1
(mean average)

Fold induction E-selectin
(mean average)

Events

PE-ICAM-1

PE-E-selectin

Resting
S1P
LPS
LPS+S1P

Pam+S1P
Pam
S1P

Fold induction ICAM-1
(mean average)

Fold induction E-selectin
(mean average)

Events

PE-ICAM-1

PE-E-selectin

Resting
S1P
LPS
LPS+S1P

Pam+S1P
Pam
S1P

Fold induction ICAM-1
(mean average)

Fold induction E-selectin
(mean average)

Events
FIGURE 3
**Figure 4**

Panel A: Immunoblots showing expression levels of ICAM-1, IL-6, IL-8, and MCP1 in HUVEC and HAEC cells under different conditions.

Panel B: Bar graph showing fold induction of IL6 secretion in HAEC cells. LPS, S1P, and LPS + S1P treatments are compared.

Panel C: Western blot analysis of COX-2 and β-tubulin expression in HUVEC cells under resting, LPS, S1P, and LPS + S1P conditions.

Panel D: Similar to Panel C, but for HAEC cells.

Panel E: Levels of 6-keto-PGF1α in HUVEC cells under different treatments.

Panel F: Levels of 6-keto-PGF1α in HAEC cells under different treatments.

Legend:
- **R**: Resting
- **S1P**: S1P treatment
- **LPS**: LPS treatment
- **LPS + S1P**: LPS + S1P treatment

*Statistical significance indicated by asterisks (e.g., *P < 0.05, **P < 0.01).
SUPPLEMENTAL FIGURE 1. Dose-response of LPS and Pam3CSK4 on the induction of inflammatory molecules. (A-B) Cells were incubated with either vehicle, or the indicated dose of LPS or S1P (1 µM) or a combination of LPS and S1P for 8h, and cell lysates were analyzed by Western blot using ICAM-1, COX-2 and β-tubulin antibodies. Images are representative of at least n = 3 experiments. (C) Flow cytometry analysis was performed using a PE-conjugated anti-human E-selectin antibody. Gating on FS and SS was applied. Histograms are representative of at least 3 independent experiments. Graphs correspond to results expressed as the fold induction increase when compared to the average of the median in resting conditions. Error bars correspond to SEM. *p < 0.05 as compared to resting conditions; # p < 0.05 for LPS + S1P vs. LPS and S1P. Pam indicates Pam3CSK4.
SUPPLEMENTAL FIGURE 2. Different S1P receptors are involved in the interplay with TLR4 in HUVEC and HAEC. Cells were pre-treated with the indicated drugs (10 μM of S1P receptor inhibitors, 100 ng/ml of PTX, 5 μM of SEW2871) for 1h before activation with LPS + S1P and lysates were analyzed by Western blot with anti-ICAM-1 (A-B) and COX-2 (D) antibodies. Equal loading was confirmed with a β-tubulin antibody. Supernatants were analyzed by ELISA to evaluate IL-6 production (C). Data represent the percentage of induction of inflammatory molecule expression by the indicated drugs with respect to the effect of treatment with LPS + S1P + vehicle (100%). Results are expressed as mean ± SEM of at least n = 3 independent experiments. JTE, JTE-013; PTX, pertussis toxin; Sew, SEW2871; Sur, suramin; VPC, VPC23019; −, vehicle.
SUPPLEMENTAL FIGURE 3. Induction of intracellular signaling cascades by LPS, S1P and LPS + S1P. (A-B) Graphs correspond to the densitometry analysis of the phosphorylated forms of p65-NF-κB, and the MAPK ERK, p38 and JNK from Figure 6A (HUVEC) and Figure 6B (HAEC), respectively. Data, normalized to the corresponding ERK1/2 band, are representative of n= 4 experiments. (C-D) Cells were incubated with either vehicle, or the indicated ligands for 8h, and cell lysates were analyzed using antibodies anti-ICAM-1, COX-2 and β-tubulin antibody. Data represent the percentage of induction of inflammatory molecule
expression by the indicated drugs with respect to the effect of treatment with LPS + S1P + vehicle (100%). Results are expressed as mean ± SEM. *p< 0.05. ALLN indicates 100 µM ALLN; PD; 50 µM PD98059; L+S, LPS+S1P; R, resting; SB, 10 µM SB203580; SN50, 50 µg/mL NF-κB SN50; SP, 10 µM SP600125.
Figure S4

A

% INTENSITY

Time (min)

B

% INTENSITY

Time (min)

C

% INTENSITY

m/z

D

% INTENSITY

m/z

E

% INTENSITY

m/z

LPS AND S1P COOPERATION ON INFLAMMATORY MOLECULES
SUPPLEMENTAL FIGURE 4. Chromatogram and mass spectrum from the liquid chromatography–tandem mass spectrometry analysis performed to measure S1P intracellular content. (A) Panel shows the extracted ion chromatogram (EIC) of the fragment m/z 264.269 from S1P standard at a concentration of 625.0 ng/mL. (B) Panel shows the extracted ion chromatogram (EIC) of the fragment m/z 264.269 from S1P obtained from the high energy function in one of the samples (LPS + S1P treatment). This chromatogram was used for quantification. (C) Panel corresponds to the mass spectrum from the high energy function of the peak at 4.15 min, S1P. (D) Panel corresponds to the mass spectrum from the high energy function of the peak at 5.45 min. (E) Panel corresponds to the mass spectrum from the high energy function of the peak at 6.36 min, sphingomyelin.