Lipopolysaccharide and Sphingosine-1-Phosphate Cooperate To Induce Inflammatory Molecules and Leukocyte Adhesion in Endothelial Cells

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Given that 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 compared with the effect of either ligand alone. Cell-type differences on E-selectin upregulation were observed. In contrast, no cooperation effect on ICAM-1 or E-selectin was observed with a TLR2/TLR1 ligand. Consistent with an increase in adhesion molecule expression, endothelial cell treatment with LPS plus S1P significantly enhanced adhesion of PBMCs under shear stress conditions compared with the effect of either ligand alone and exhibited comparable levels of cell adhesion strength as those after TNF-α treatment. Moreover, LPS and S1P cooperated to increase the expression of proinflammatory molecules such as 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 plus S1P treatment of HUVEC and human aortic endothelial cells and cell-type differences on p38 and NF-κB activation. Moreover, pharmacological and small interfering RNA 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. Sphingosine kinase activity induction upon LPS plus S1P treatment suggests S1P– Sphingosine kinase axis involvement. In summary, LPS and S1P cooperate to increase proinflammatory molecules in endothelial cells and, in turn, to augment leukocyte adhesion, thus exacerbating S1P-mediated proatherosclerotic/ proinflammatory properties. The Journal of Immunology, 2012, 189: 5402–5410.

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 the actions of S1P are mediated by the activation of receptors, initially known as endothelial differentiation genes and now designated as S1P1–5, which are high-affinity receptors coupled to G proteins (αi/o, 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, and so forth, and interference in 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 the pathophysiological role of S1P, even though it has been suggested that S1P could explain in part the atheroprotective effects of high-density lipoproteins, both S1P-mediated anti-atherogenic and proatherogenic effects have been reported in the vascular system (8–10). In endothelial cells, S1P promotes the expression of adhesion molecules associated with angiogenesis and atherosclerosis processes and the expression of inflammation-related genes, although it also promotes anti-atherogenic effects (8–11).

TLRs are sensors of microbial components that play an important role in innate immunity (12) and the pathogenesis of several inflammatory diseases such as systemic lupus erythematosus, sepsis, and 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 atherosclerosis (14). In endothelial cells, TLR activation has been reported to promote lipid uptake and adhesion of leukocytes in 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, human aortic endothelial cells (HAEC), endothelial cell growth medium-2 (endothelial medium supplemented with growth factors and cytokines, FBS, nuclease-free water, HEPES, and antibiotics) were purchased from Clonetech-Lonza (Walkersville, MD). M199, TRIZol, and Lipofectamine 2000 were from Invitrogen (Carlsbad, CA). Biocoll gradients were from Biochrom KG (Berlin, Germany). Pcm,CSK4 was from InvivoGen (San Diego, CA). LPS from Escherichia coli type 0111:B4 (no. L2630), S1P (no. S9666), SP600125, anti-human β-tubulin Ab (no. T7816), and pertussis toxin (PTX) were purchased from Sigma (St. Louis, MO). S1P was dissolved in methanol, following the manufacturer’s instructions; LPS and Pcm,CSK4 were dissolved in endotoxin-free water. Anti-human ICAM-1 (no. sc-1511) and cyclooxygenase-2 (COX-2) (no. sc-1745) Abs for the phosphorylated forms of ERK, JNK, and p38 MAPK. Equal loading across the gel was confirmed using anti-β-tubulin or ERK1/2Abs. 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 either with PE-conjugated anti–ICAM-1 or PE-conjugated anti–E-selectin Abs or with the corresponding isotype-specific control Ab. Flow cytometry analysis was performed in a Beckman Coulter device (Epics XL-MCL). Data were 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, PBM C (2 × 10⁷/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 ImageJ 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 Abs. Images were obtained with a fluorescence microscope coupled to a digital camera (DXM1200C Nikon Eclipse 90i), and fluorescence intensity was estimated using ImageJ 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°C. Migrated cells were fixed with 3% p-formaldehyde and stained with 0.1% crystal violet. Images were obtained with a Nikon eclipse TS-100 microscope coupled to a digital camera (Nikon), and cell counting was performed using ImageJ software.

Ab arrays and ELISA

Cells were stimulated with the corresponding ligand for 8 h at 37°C 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-PGF₁α secretion by ELISA following the manufacturer’s protocol.

siRNA experiments

Cells were transfected with Lipofectamine 2000 (HEVC) or by nucleofection (HAEC) following the manufacturer’s protocol. Validated siRNA duplexes specific for S1P₁ and S1P₃ were as described (20). Quantitative PCR was performed to confirm downloading of human S1P receptors as described (20). Transfected cells were activated, and ICAM-1 and COX-2 were analyzed by Western blot.

SIP quantification by liquid chromatography–tandem mass spectrometry analysis

HAEC were stimulated for 75 min at 37°C 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 methanol/water (95:5, v/v) and kept at –80°C until analysis. Ultraperformance liquid chromatography–tandem mass spectrometry interfaced with a time-of-flight mass spectrometer was performed as described (24), with slight modifications, using an Acquity UPLC System and SYNAT HDMS G2 (Waters, Milford, MA). A two-solvent gradient elution was used for compound separation using an Acquity BEH C18 column (1.7 μm × 2.1 mm × 30 mm, temperature 30°C), as follows: 1) 0–1 min, 100% A + 0% B; 2) 1.0–4.5 min, 0% A + 100% B; 3) 4.5–6.0 min, 0% A + 100% B (isocratic); and 4) 6.0–8.0 min, 100% A + 0% B. Solvent A consisted of methanol/water/formic acid (50:50:0.1, 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, and the injection volume was 7.5 μl.
Results are expressed as mean ± SEM. Data were analyzed either 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 \( p < 0.05 \).

**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, S1P₁ being the most abundant and significantly more highly expressed in HAEC than in 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 after LPS plus S1P stimulation in HUVEC compared with cells treated with either ligand alone (Fig. 1C), and the cooperation effect was observed at 8 h after treatment and lasted at least for 12 h (Fig. 1C). The effect was observed in the range 1–5 \( \mu \)g/ml of LPS (Supplemental Fig. 1A), and 1 \( \mu \)g/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 on 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, 2B). In contrast, the TLR2/TLR1 ligand Pam₃CSK₄ did not show any cooperative effect with S1P even at higher doses either in HUVEC (Fig. 2G) or in HAEC (Fig. 2H).
Together, the 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 upregulation 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 plus S1P compared with cells treated with either ligand alone (Fig. 3A). Moreover, PBMCs that adhered to HAEC monolayers treated with LPS plus S1P were highly resistant to laminar flow compared with cells treated with either ligand alone, and many mononuclear cells still remained attached at physiologically relevant shear conditions (Fig. 3B), indicating that LPS and S1P cooperate to increase the strength of PBMC adhesion to endothelial cells. Furthermore, LPS plus S1P treatment exhibited comparable levels of cell adhesion strength as those of TNF-α treatment (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 upregulation of ICAM-1 expression. Consistent with the increase in the number of attached PBMCs and the adhesion strength observed in Fig. 3A and 3B, cells treated with LPS plus S1P expressed higher levels of ICAM-1 [17,281 ± 4,266 arbitrary units (a.u.)] than cells treated with either LPS (7,994 ± 1,048 a.u.) or S1P (1,241 ± 387 a.u.) (Fig. 3C). In contrast, LPS and S1P showed no cooperative effect on other cell functions, as LPS inhibited S1P-mediated chemotaxis of endothelial cells (Fig. 3D, 3E). The inhibiting effect of LPS on S1P-induced migration cannot be explained by an LPS-induced downregulation of S1P1, as LPS showed no effect on S1P1 receptor mRNA levels in real-time RT-PCR experiments (resting, 6.64 ± 0.11 a.u.; LPS treatment for 2 h, 6.90 ± 0.12 a.u.; LPS 4 h, 6.38 ± 0.09 a.u.). In contrast, S1P did not affect TLR4 levels (resting, 2.33 ± 0.18 a.u.; S1P 2 h, 1.91 ± 0.66 a.u.; S1P 4 h, 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 upregulation of proinflammatory mediators such as IL-6, COX-2, and prostacyclin**

Next, we explored whether LPS and S1P cooperate to induce the expression of proinflammatory cytokines using a human inflamm-
mation Ab array. Cell treatment with LPS or S1P for 8 h induced the expression of IL-6, IL-8, and ICAM-1, and LPS plus S1P further increased ICAM-1 expression, consistent with data from Figs. 1 and 2, and IL-6 secretion, as observed with a human inflammation Ab array (Fig. 4A). TNF-α was not induced by either LPS or LPS plus S1P (Fig. 4A). Quantification of IL-6 production by ELISA showed that LPS plus S1P induced a significantly higher production of IL-6 than that of each ligand alone in HAEC (Fig. 4B), demonstrating a cooperation of LPS and S1P on the expression of this proinflammatory cytokine.

Because the proinflammatory 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 plus S1P induced a significantly higher production of IL-6 than that of each ligand alone in HAEC (Fig. 4B), demonstrating a cooperation of LPS and S1P on the expression of this proinflammatory cytokine.

Because the proinflammatory 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 plus S1P induced a further increase (Fig. 4C). Similar results were observed in HAEC, and the cooperative effect was observed as early as 4 h 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-PGF1α secretion. Consistent with a cooperation effect on COX-2 expression, the levels of secreted 6-keto-PGF1α were significantly higher in cells treated with LPS plus S1P compared with the effect of either ligand alone (Fig. 4E).

Altogether, these results demonstrate that LPS and S1P cooperate to upregulate the expression of proinflammatory 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 plus S1P for 8 h, and cell lysates were analyzed by Western blot. The cooperative effect of LPS plus S1P on ICAM-1 induction was sensitive to PTX, which inhibits Gia/o proteins predominantly coupled to S1P1 but also S1P2/3, and to W146, a S1P1 antagonist, but was not affected by JTE or 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). In contrast, in HAEC the effect on ICAM-1 induction was PTX-sensitive and significantly inhibited by VPC23019 (an 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, 5D), suggesting the involvement of S1P3 although differences in PTX sensitivity were observed.

Cell treatment with LPS plus S1P activates NF-κB and MAPK routes (Fig. 6A, 6B). Notably, in HUVEC and HAEC, LPS plus S1P induced the phosphorylation of ERK in a synergistic manner, as the effect was higher than that obtained by the sum of the effect of each ligand alone (Supplemental Fig. 3A, 3B), suggesting that the ERK/MAPK pathway might be a cross-road signaling point. Moreover, in HUVEC, p38 might also serve as a point of confluence (Supplemental Fig. 3A). Next we tested the effect of different signaling inhibitors on the cooperative effect on ICAM-1 blockade of the NF-κB route with ALLN, a proteasome inhibitor, which significantly inhibited the induction of ICAM-1 expression by LPS plus S1P in HUVEC and HAEC (Fig. 6C, 6D, Supplemental Fig. 3C, 3D). Moreover, SN50, which prevents NF-κB translocation into the nucleus, partially inhibited ICAM-1 induction (Fig. 6C, 6D). As shown in Fig. 6C and 6D, the effect on ICAM-1 induction was statistically significantly reduced with the ERK inhibitor PD98059 in HUVEC and to a lesser 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, 6D) and was statistically significantly inhibited by SB203580 and by SB600125 in HUVEC and by SB203580 in HAEC (Fig. 6C, 6D), thus pointing to the involvement of different MAPKs.

Because LPS has been described to increase S1P intracellular levels by SphK-1 (2), the S1P intracellular content was analyzed by mass spectrometry in HAEC. LPS induced a small but not statistically significant increase of S1P endogenous levels (Fig. 4).

**FIGURE 4.** LPS and S1P cooperate to upregulate 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 Materials and Methods. Images are representative of two independent experiments. Squares, 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 compared with resting levels and are expressed as mean average ± SEM of n = 4 experiments in duplicate. (C and 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 Abs. Densitometry data are expressed as in Fig. 1. (E and F) Supernatants were analyzed by ELISA for the production of 6-keto-PGF1α after 8 h of activation. Data and images are representative of at least three experiments in duplicate. *p < 0.05 (compared with resting conditions), †p < 0.05 (LPS + S1P versus LPS and S1P).
Discussion

The current study reveals that exposure of endothelial cells to LPS and S1P leads to a cooperative induction of proinflammatory molecules that have a strong impact on leukocyte adhesion under physiologic flow conditions. This might have relevance in inflammatory processes and vascular pathophysiology. 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 triggers a series of molecular and cellular events that may contribute to inflammatory vascular diseases. LPS plus S1P cooperatively increased the expression of adhesion molecules and other inflammatory molecules thus enhancing the proinflammatory/proadhesive 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 upregulation suggests that the cooperation between LPS and S1P is relevant not only to adhesion but also to endothelial rolling. Moreover, the 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, to our knowledge, that exposure of endothelial cells from arterial origin to LPS plus S1P cooperatively increased both adhesion molecule expression and adhesion of mononuclear cells 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 plus 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, which 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, as there exists evidence for both proatherogenic and anti-inflammatory roles of S1P.

Our results would argue for the dual role of S1P in the pathogenesis of atherosclerosis, which is not unique to S1P, but rather a shared feature of many (pro)inflammatory mediators. However, several questions remain to be answered. First, it is not yet known whether the cooperative effect of LPS and S1P in proinflammatory processes is restricted to adhesion molecules. Second, it needs to be addressed whether the cooperation is restricted to the arterial bed or also to the venous one. Third, the cooperation may be a result of the interaction of different S1P receptors and regulators as well as intracellular signaling cascades leading to the cooperative effects. Together, the data demonstrate involvement of different S1P receptors and regulators as well as intracellular signaling cascades in the cooperation with LPS to induce proinflammatory molecules in endothelial cells from venous and arterial origin.

Figure 6. Different signaling cascades are involved in the interplay of TLR4 and S1P receptors in HUVEC and HAEC. (A and B) HUVEC and HAEC were incubated with either vehicle or the indicated ligands for the indicated times, and cell lysates were analyzed using Abs for the phosphorylated forms of p65 NF-κB and the MAPK ERK p38 and JNK. Equal loading was confirmed with an anti-ERK Ab. Images are representative of four experiments in duplicate. See Supplemental Fig. 3 for ratiometric analysis. (C and D) Cells were pretreated with the indicated drugs for 1 h before activation with LPS + S1P, and lysates were analyzed by Western blot with anti–ICAM-1 and COX-2 Abs. Images are representative of at least three experiments in duplicate. PD, 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 milligrams of protein. Data, expressed as fold induction of S1P intracellular content (mean ± SEM) relative to the values obtained after exogenous S1P treatment, are representative of n = 3 experiments. *p < 0.05. (F and 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 compared with 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 (compared with untreated cells).
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 nanomolar, arguing in favor of an S1P receptor-mediated and physiologically relevant effect.

Our data provide the first demonstration, to our knowledge, of LPS and S1P cooperation to induce the expression of proinflammatory molecules such as 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, such as 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). In contrast, 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, as 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 plus S1P induces a cooperative upregulation of the proinflammatory 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. S1P1/2 are the predominant receptors in endothelial cells, and, notably, 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). In contrast, S1P3, but also S1P2, are involved in the cooperation effect in endothelial cells from aortic origin. Notably, S1P1/2 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 proadhesive 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 monocytes/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 plus S1P induced the synergistic activation of ERK, arguing for a role of that pathway in the cooperative effect. Cell type-specific effects were observed, as p38 and NF-κB synergistic induction by dual stimulation is observed in HUVEC, whereas the effect is additive in HAEC. At the level of proinflammatory 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, whereas 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 proadhesive effects in endothelial cells are mediated by NF-κB (9), a master gene regulator of many proinflammatory 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, as LPS plus S1P induced a significant increase of S1P endogenous levels compared with 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/SphK-1 signaling level. The SphK activity, detected in endothelial cells upon LPS plus 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 SphK-1 (39). However, the contribution of TNF-α-induced endogenous S1P could not be ruled out because LPS-induced TNF-α expression and TNF-α–mediated activation of SphK-1–S1P signaling has been shown (2, 11). The lack of TNF-α induction observed in cytokine arrays after 8 h of treatment with LPS plus 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 in this study with LPS plus 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 RBCs activation, or after platelet aggregation/activation occurring in active cardiovascular disease states both on a chronic and acute basis (40, 41). On the basis of these data, we propose that a two-signal paradigm might be required for an exacerbated inflammatory response, which could underlie the proinflammatory mechanism leading to disease.

In summary, our data underscore cooperation between LPS and S1P that leads to an increase in leukocyte adhesion and proinflammatory responses in endothelial cells, thus intensifying S1P-mediated proinflammatory/proadhesive properties, which might have some consequences in vascular pathophysiology. 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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Disclosures

The authors have no financial conflicts of interest.
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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.
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 5.53 min. (E) Panel corresponds to the mass spectrum from the high energy function of the peak at 6.26 min, sphingomyelin.