Protection of LPS-Induced Murine Acute Lung Injury by Sphingosine-1-Phosphate Lyase Suppression

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A defining feature of acute lung injury (ALI) is the increased lung vascular permeability and alveolar flooding, which leads to associated morbidity and mortality. Specific therapies to alleviate the unrelenting vascular leak in ALI are not currently clinically available; however, our prior studies indicate a protective role for sphingosine-1-phosphate (S1P) in animal models of ALI with reductions in lung edema. As S1P levels are tightly regulated by synthesis and degradation, we tested the hypothesis that inhibition of S1P lyase (S1PL), the enzyme that irreversibly degrades S1P via cleavage, could ameliorate ALI. Intratracheal instillation of LPS to mice enhanced S1PL expression, decreased S1P levels in lung tissue, and induced lung inflammation and injury. LPS challenge of wild-type mice receiving 2-acetyl-4(5)-[1(R),2(S),3(R),4-tetrahydroxybutyl]-imidazole to inhibit S1PL or S1PL^{+/-} mice resulted in increased S1P levels in lung tissue and bronchoalveolar lavage fluids and reduced lung injury and inflammation. Moreover, down-regulation of S1PL expression by short interfering RNA (siRNA) in primary human lung microvascular endothelial cells increased S1P levels, and attenuated LPS-mediated phosphorylation of p38 mitogen-activated protein kinase and I- κ B, IL-6 secretion, and endothelial barrier disruption via Rac1 activation. These results identify a novel role for intracellularly generated S1P in protection against ALI and suggest S1PL as a potential therapeutic target.

Keywords: intracellular sphingosine-1-phosphate; sphingosine-1-phosphate lyase; IL-6; transendothelial resistance; acute lung injury

Sphingosine-1-phosphate (S1P), a naturally occurring, bioactive sphingo phospholipid, is present in plasma and tissues with three- to four-times higher levels in serum than in plasma (1). Extracellular actions of S1P are mainly through its G protein-coupled receptors (S1P₁₋₆) expressed on plasma membrane of cells (2–6). In addition to its extracellular action, S1P can signal intracellularly (7–10). Intracellular S1P generated by the photolysis of caged S1P raised the intracellular free Ca²⁺ concentration in HEK-293, SKNMC, and HepG2 cells, independent of S1P receptors (7) and induced DNA synthesis (8). In adult rat dorsal root ganglion neurons, intracellular S1P generated from ceramide regulated membrane excitability (10). Since our original description of the barrier regulatory properties of S1P (11), a number of *in vitro* and *in vivo* reports have highlighted the prominent effects of S1P on barrier integrity (9, 11–13),

CLINICAL RELEVANCE

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cytokine release (14), migration (15), and neutrophil infiltration in lung alveolar spaces, and inflammatory histologic changes by endotoxin (16, 17). These findings suggest S1P as a major barrier-protective agent responsible for maintenance of vascular barrier integrity *in vitro* and *in vivo*, processes relevant to lung inflammation.

Although cellular targets of S1P have not been identified and characterized, a recent study has identified histone deacetylases 1 and 2 as potential intracellular targets (18). We have shown previously that intracellular S1P can be generated from extracellular S1P by actions of lipid phosphate phosphatases (LPPs) and sphingosine kinases (SphKs) in human lung endothelial cells (ECs) (19); however, the role of intracellular S1P in regulation of ECs functions is not well defined. Accumulation of intracellular S1P is a balance between its formation from sphingosine catalyzed by sphingosine kinases (SphKs) 1 and 2 and metabolism mediated by S1P phosphatases, LPPs and S1P lyase (S1PL). S1PL irreversibly converts S1P to phosphoethanolamine and hexadecenal, which links the metabolic pathways of sphingo- and glycerophospholipids (20–22). Recent evidence suggests a role for S1PL in normal development, reproduction, cell survival, cancer and immunity (23–25). S1PL function seems to be critical for mammalian survival as homozygous *S1PL* (*Sgpl1*) knockout mice do not survive beyond a couple of weeks after birth and null mice exhibit abnormalities in vascular and other tissues (25, 26). 2-Acetyl-4-tetrahydroxybutylimidazole (THI) treatment increases S1P levels in lymphoid tissues of mice by inhibiting S1PL activity, and this treatment causes accumulation of mature cells in the thymus and loss of lymphocytes from the lymph and blood (27). The role of products of S1PL in regulating physiological responses such as mitogenesis remains controversial (28).

Acute lung injury (ALI) is a refractory lung disease occurring in response to diverse inciting stimuli (sepsis, acid aspiration, trauma, etc.), and is characterized by severe hypoxemia and an unacceptably high mortality (30–50%) (29, 30). Although specific mechanisms involved in ALI remain elusive, a defining feature of ALI is the increased lung vascular permeability, alveolar flooding causing increased morbidity and mortality (29–31). Recent studies in SphK1-deficient mice show marked enhancement of pulmonary edema formation and cytokine production in response to LPS (32, 33), suggesting

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a protective role of SphK1 and possibly intracellular S1P in LPS-induced murine model of ALI. The current study, for the first time, demonstrates that increase of S1P levels by reducing S1PL expression or activity protects LPS-induced cytokine release, endothelial barrier disruption, and inflammation in a murine model of ALI and human lung microvascular ECs (HLMVECs). Furthermore, down-regulation of S1PL with short interfering RNA (siRNA) attenuated LPS-induced p38 mitogen-activated protein kinase (MAPK)/NF- κ B signal transduction, IL-6 secretion, and endothelial permeability, whereas overexpression of S1PL wild type (WT) enhanced LPS-mediated p38 MAPK/NF- κ B activation, IL-6 production, and permeability in HLMVECs. Thus, the current study has identified S1PL as a target of endotoxin-mediated lung injury, and characterizes molecular mechanisms linking the protecting role of intracellular S1P in ameliorating inflammatory response and barrier disruption by LPS in *in vitro* and *in vivo* models of ALI.

MATERIALS AND METHODS

EC Culture

HLMVECs were purchased from Lonza (Walkersville, MD). Passages between 5 and 7 were grown as monolayers in Endothelial Cell Growth Medium-2MV complete media with 10% FBS, 100 U/ml penicillin and streptomycin in a 37°C incubator under 5% CO₂-95% air atmosphere. Cells from T-75 flasks were detached with 0.25% trypsin and resuspended in fresh complete medium and cultured in 35- × 60-mm diameter dishes, glass chamber slides, or electrical cell substrate impedance sensing system (ECIS; Applied Biophysics, Troy, NY) chamber slides with attached golden microelectrodes.

Lung Histology

Lungs were formalin fixed and embedded in paraffin. Sections (5 μ m) were stained with hematoxylin and eosin for histological evaluation. Cells in lung parenchyma were counted on nonoverlapping high-power fields at 600 $\times$ magnification to calculate the number of infiltrating polymorphonuclear leukocytes.

Intratracheal or Intraperitoneal Challenge of LPS

LPS was administered intratracheally or intraperitoneally to 8- to 10-week-old C57BL/6 WT or S1PL^{+/-} mice in 129SV background. Mice were anesthetized with 3–5 ml/kg of a mixture of 25 mg/kg of ketamine and 2.5 ml of xylazine. LPS (5 mg/kg body weight) in PBS or PBS alone was administered intratracheally. In some experiments, mice were challenged with LPS (15 mg/kg body weight) intraperitoneally. After 24 hours, bronchoalveolar lavage (BAL) was performed with an intratracheal injection of 2 ml of PBS solution followed by gentle aspiration. The recovered fluids were processed for protein concentration, IL-6, and S1P measurement. Lungs from challenged mice were collected for histologic evaluation by hematoxylin and eosin staining. Plasma was collected and S1P levels were measured by liquid chromatography–tandem mass spectrometry (LC-MS/MS).

THI Administration

After 0.5 hour of LPS intratracheal challenge, THI (0.5 ml in 0.05 mg/ml H₂O) or H₂O alone were administered orally three times per day by gavage to C57BL/6 mice for 2 days, and, at 48 hours of LPS challenge, plasma, BAL fluids, and lung tissues were collected.

Lipid Extraction and Sample Preparation for LC-MS/MS Analysis of S1P

Cellular, plasma, bronchoalveolar lavage fluids, or extracellular lipids were extracted by a modified Bligh and Dyer procedure under acidic conditions using 0.1 M HCl and C17-S1P (40 pmol) and C17-Sph (30 pmol), which were added as internal standards during the lipid extraction step. The lipid extracts were dissolved in ethanol (200 μ l), and aliquots were analyzed for total lipid phosphate and then subjected to LC-MS/MS for quantification of S1P, as described previously (34).

Measurement of Transendothelial Resistance

HLMVECs were grown to approximately 100% confluence in six-well plates with attached golden microelectrodes. Changes in transendothelial resistance (TER) were measured in an electrical cell substrate impedance sensing system, as described earlier (35).

Statistical Analysis

All results were subjected to statistical analysis using one-way ANOVA and, whenever appropriate, analyzed by Student-Newman-Keuls test. Data are expressed as means ($\pm$ SEM) of triplicate samples from at least three independent experiments, and level of significance was taken as a *P* value less than 0.05.

RESULTS

Genetic Deletion of S1PL Attenuates LPS-Induced Lung Inflammation

To characterize the role of S1PL in modulating LPS-mediated lung injury, we used the S1PL^{+/-} (heterozygous) mice, as deletion of both alleles of *sgpl* gene in mice results in vascular development defects and mortality within 4 weeks of birth (28). S1PL mRNA and protein expression in S1PL^{+/-} mice were approximately 50% of WT mice, with a reduction in S1PL activity of lung homogenates from S1PL^{+/-} mice (data not shown). Analysis of lung tissue, BAL fluids, and plasma for S1P levels by LC-MS/MS revealed a small but significant increase of S1P levels in lung tissue and BAL fluids, but not in plasma of S1PL^{+/-} mice, as compared with WT mice (Figures 1A–1C).

To assess whether S1PL plays a role in LPS-induced lung injury, control WT and S1PL^{+/-} mice were instilled intratracheally with vehicle or LPS (5 mg/kg body weight). The animals were allowed to recover for 24 hours after LPS instillation, whole lungs were lavaged, and BAL fluids were collected and analyzed. LPS challenge of WT mice significantly increased IL-6 and protein levels in BAL fluids relative to the vehicle-treated groups (Figures 1D and 1E); however, the increase of IL-6 and total protein in BAL fluid mediated by LPS was significantly lower in S1PL^{+/-} mice as compared with LPS-challenged WT mice (Figures 1D and 1E). Next, we examined histologic characteristics of the lungs from WT and S1PL^{+/-} mice with and without LPS challenge. Using hematoxylin and eosin staining, no significant differences in lung morphology between WT and S1PL^{+/-} were observed; however, influx of inflammatory cells into alveolar space and injury in response to LPS was attenuated in S1PL^{+/-} mice compared with WT mice (Figure 1F). Quantification of 4'-6-diamidino-2-phenylindole (DAPI)-positive cells revealed significantly lower infiltration of cells in S1PL^{+/-} heterozygous mice exposed to LPS compared with S1PL^{+/+} WT mice (Figure 1G). These results suggest that deletion of single *sgpl* allele modulates S1P levels in lung tissue and BAL fluids, and partially protects mice against LPS-induced lung injury.

Administration of THI Increases S1P Levels and Reduces LPS-Induced Inflammation in Mice

To investigate further the role of S1PL in LPS-induced lung inflammation and injury, we evaluated the effect of THI, an inhibitor of S1PL that has been shown to increase S1P levels in lymphoid tissues of mice and induce lymphopenia (27). Administration of THI (0.5 ml in 0.05 mg/ml water) orally (three times per day) for 2 days after control or LPS instillation (5 mg/kg body weight) 30 minutes after challenge (Figure 2A) elevated S1P levels by two- to threefold in lung tissue and BAL fluids without significantly altering the plasma S1P (Figures 2B–D). Furthermore, THI treatment after LPS challenge attenuated IL-6 release and infiltration of neutrophils into alveolar space as

Figure 1. Sphingosine-1-phosphate lyase (S1PL)^{+/-} mice exhibit decreased inflammatory responses to LPS in a murine model of acute lung injury (ALI). C57BL/6J-129/Sv control ($n = 6$) and S1PL^{+/-} mice ($n = 6$) with or without intratracheal (i.t.) instillation of LPS (5 mg/kg body weight) for 18 hours. S1P levels in lung tissue (A), bronchoalveolar lavage (BAL) fluids (B), or plasma (C) were quantified by liquid chromatography–tandem mass spectrometry (LC-MS/MS). BAL fluids from control and S1PL^{+/-} mice were analyzed for IL-6 (D) and total protein (E). Lung tissue sections from control and S1PL^{+/-} mice with or without LPS challenge, stored in formalin, were processed for staining with hematoxylin and eosin for infiltration of cells into alveoli (F). Shown is a representative staining of the lung tissue from three independent experiments. (G) Fold change in polymorphonuclear leukocyte infiltration in the lung after exposure of control and S1PL^{+/-} animals to PBS or LPS (5 mg/kg body weight) for 18 hours. Values are means ($\pm$ SE) for three independent experiments.

compared with control mice (Figures 2E and 2F). Histologic examination of the lungs from THI-treated mice revealed attenuation of extensive inflammatory cells infiltration induced by LPS (Figure 2G). Quantification of infiltrating cells revealed an approximately 2.5-fold increase in cells after LPS challenge, and THI administration significantly reduced the infiltration of cells to approximately 1.2-fold, compared with controls, in lung parenchyma (Figure 2H). Taken together, these results suggest that either deletion or blocking S1PL activity *in vivo* to modulate S1P levels exerts a potential beneficial effect on LPS-induced lung inflammation and injury.

LPS Stimulates S1PL Expression and Decreases S1P Levels in Mouse Lung

The protective effect of either S1PL gene deletion or inhibition of S1PL activity by THI in LPS-induced injury murine models suggests a potential link between LPS-mediated modulation of S1P levels and injury. As S1P levels are regulated by its synthesis and degradation, the effect of LPS challenge on S1PL expression, activity, and S1P levels was determined. Intratracheal instillation of LPS (5 mg/kg body weight; 24 h) reduced S1P levels in lung tissue (S1P: vehicle, 296 ± 24 fmol/nmol lipid Phosphorus (P); LPS [intratracheal], 138 ± 16 fmol/nmol lipid P), as well as in plasma (S1P: vehicle, $1,126 \pm 36$ fmol/nmol lipid P; LPS [intratracheal], 825 ± 29 fmol/nmol lipid P) (Figures 3A and 3B). LPS challenge, either intraperitoneal or intratracheal, enhanced S1PL mRNA expression (data not shown) and protein levels approximately twofold (Figures 3C and 3D). These results show a definitive link between increased S1PL expression and decreased S1P levels in response to LPS challenge in the mouse lung.

LPS Modulates Intracellular S1P Levels and Expression of S1PL in HLMVECs

To determine the role of S1PL in lung inflammation and injury, HLMVECs were exposed to LPS (100 ng/ml) in Endothelial Cell Basal Medium-2 (EBM-2) supplemented with 1% FBS for 3 hours, which significantly reduced intracellular S1P levels ($\sim 30\%$) compared with control cells (Figure 4A). Exposure of HLMVECs to LPS for 3 hours had no effect on SphK1 and SphK2 protein expression and activity (data not shown), but increased S1PL mRNA and protein expression (Figures 4B and 4C) (~ 4.1 -fold and ~ 1.6 -fold, respectively). In addition, S1PL activity in cell lysates after LPS challenge was enhanced approximately threefold as compared with control cells (Figure 4D). These results show that LPS increases expression of S1PL and decreases intracellular S1P levels in HLMVECs similar to mouse lung tissue.

Involvement of Toll-Like Receptor 4 and Myeloid Differentiation Primary Response Gene 88 (MyD88) in LPS-Induced IL-6 Release in HLMVECs

To determine the mechanism(s) by which S1PL regulates LPS-induced lung inflammation and injury, we further characterized LPS-mediated cytokine release. LPS, in a time-dependent manner, stimulated IL-6 secretion (see Figure E1A in the online supplement), and optimal IL-6 release by LPS was observed at a concentration of 100 ng/ml (data not shown). The LPS mediated IL-6 secretion was partially dependent on Toll-like receptor 4 (TLR4), as down-regulation of TLR4 with siRNA (50 nM, 72 h) reduced IL-6 secretion by approximately 60% and TLR4 protein expression by approximately 72% (Figures E1B

Figure 2. After treatment with S1PL inhibitor, THI, ameliorates LPS-induced inflammatory responses in mice. (A), THI (0.05 mg/ml in 500 μ l) was orally administered at 0.5, 4, 7, 24, 27, and 30 hours after intratracheal instillation of LPS (6–8 mice/subgroup). (B) S1P levels in lung tissue, (C) BAL fluids, and (D) plasma were measured by LC-MS/MS. (E) IL-6 levels in BAL fluids were measured by ELISA kit. (F) Cell numbers in BAL fluids from control and THI-treated animals. (G) Lung tissue sections were stained with hematoxylin and eosin for infiltration of cells. Shown is a representative stained lung tissue from three independent experiments. (H) The fold change of polymorpholeukocyte infiltration in the lung after exposure to PBS or LPS in the presence or absence of THI quantified by high-power microscopy ($P < 0.01$ versus PBS). Values are means ($\pm$ SE) for three independent experiments.

and E1C). Furthermore, pretreatment of cells with an MyD88 (a TLR4 adaptor protein) peptide inhibitor (100 μ M) for 24 hours almost completely blocked LPS-induced IL-6 secretion (Figure E1D). These results confirm a role for TLR4 and MyD88 in LPS-induced IL-6 secretion signal transduction in HLMVECs.

Role of S1PL and SphKs in LPS-Mediated IL-6 Secretion in HLMVECs

Next, we investigated the role of S1PL and SphKs on LPS-induced IL-6 secretion. HLMVECs were transfected with vector control or S1PL WT plasmid before LPS challenge (100 ng/ml, 3 h). Transfection of HLMVECs with S1PL WT plasmid (1 μ g/ml, 48 h) increased expression of the protein by approximately 11-fold and potentiated LPS-induced IL-6 secretion by approximately 3.1-fold compared with control cells challenged with LPS (Figure 5A). In contrast, down-regulation of S1PL with S1PL siRNA (50 nM) for 72 hours decreased LPS-mediated IL-6 release by approximately 50% without altering the basal IL-6 release (Figure 5B). As SphKs generate S1P from sphingosine, we also evaluated the effect of knockdown of SphK1 and SphK2 on LPS-induced IL-6 production. Down-regulation of SphK1 or SphK2 with siRNA (50 nM, 72 h) significantly elevated IL-6 generation in HLMVECs (Figure E2A). Inhibition of SphK activity with the inhibitor, SphKi,

resulted in increased IL-6 production (Figure E2B). These results suggest that modulation of S1P levels in cells by knockdown or overexpression of S1PL, or by knockdown of SphKs, regulates LPS-induced IL-6 secretion in HLMVECs.

To further define the role of S1PL, we determined S1P levels in cells and medium of scrambled and S1PL siRNA-treated HLMVECs in the presence or absence of sodium vanadate (1 mM), which inhibits ecto-LPP activity (34). In the absence of sodium vanadate, S1P was not detectable in the medium of scrambled or S1PL siRNA transfected cells by LC-MS/MS in the absence of sodium vanadate. However, addition of sodium vanadate (1 mM) to scrambled or S1PL siRNA transfected cells increased S1P in the medium as well as in cells (Figures 5C and 5D). These results show the ability of human lung ECs to release intracellularly generated S1P into the medium, which could act via S1P receptors.

Comparison of Exogenously Added S1P versus Intracellularly Generated S1P in Ameliorating LPS-Induced IL-6 Secretion in HLMVECs

To address the role of exogenously added versus intracellularly generated S1P in ameliorating LPS-induced IL-6 production, HLMVECs were transfected with scrambled or S1PL siRNA (50 nM, 72 h) before LPS challenge (100 ng/ml) for 3 hours. As shown in Figure E2, exogenously added sphingosine (1 μ M) or

Figure 3. Effect of LPS on S1PL expression and S1P levels in lung tissue and plasma. C57BL/6J-129/Sv control ($n = 6$) and $S1PL^{+/-}$ mice ($n = 6$) were challenged with LPS intratracheally (5 mg/kg) for 18 hours. S1P levels in lung tissues (A) and plasma (B) were quantified by LC-MS/MS. (C) LPS was administered intraperitoneally (15 mg/kg) for 24, 48, and 72 hours and cell lysates from control and LPS-treated lung tissues were analyzed for S1PL protein expression by Western blotting. (D) Cell lysates from control and lung tissues intratracheally challenged with LPS (5 mg/kg) were probed for S1PL expression by Western blotting. Shown are representative blots.

S1P (1 μ M) attenuated LPS-induced IL-6 secretion by approximately 11% and approximately 21%, respectively; however, down-regulation of S1PL with S1PL siRNA reduced LPS-induced IL-6 secretion by approximately 50% compared with scrambled siRNA transfected cells (Figure E3). These results suggest that intracellularly generated S1P is more effective in ameliorating LPS-induced IL-6 secretion than exogenously added sphingosine or S1P.

S1PL Regulates LPS-Induced IL-6 Secretion through p38 MAPK and NF- κ B Signal Transduction

LPS stimulated NF- κ B and ERK1/2 and p38 MAPK (Figures E4A and E4B), confirming earlier studies in other cell types (35–37). Inhibition of p38 MAPK and NF- κ B with Bay11-7082 and SB203580, but not ERK with PD98059, partly blocked LPS-mediated IL-6 secretion in HLMVECs (Figures E4C and E4D). Next, we investigated the role of S1PL in p38 MAPK and NF- κ B activation. LPS (100 ng/ml, 2 h) stimulated p38 MAPK and I- κ B phosphorylation that was attenuated by S1PL siRNA (50 nM, 72 h) without affecting the expression of total p38 MAPK or actin (Figure 6A). To define further the specificity of intracellularly generated S1P in modulating LPS-induced signal transduction, HLMVECs were challenged with TNF- α (20 ng/ml) that enhanced p38 MAPK and I- κ B phosphorylation, whereas down-regulation of S1PL by siRNA did not modulate the TNF- α response (Figure 6B). Conversely, overexpression of S1PL WT (1 μ g cDNA plasmid/ml, 48 h) potentiated LPS-mediated phosphorylation of p38 MAPK and I- κ B (Figure 6C). These results show that altering S1P levels by down-regulation or

Figure 4. LPS regulates S1PL expression and activity in human lung microvascular endothelial cells (HLMVECs). HLMVECs (~90% confluent) were exposed to medium containing 1% FBS or LPS (100 ng/ml) for 3 hours. (A) Intracellular S1P levels were quantified by LC-MS/MS. (B) Total RNA was isolated and S1PL mRNA levels were quantified by real-time RT-PCR. (C) Cell lysates from LPS-challenged HLMVECs were probed for S1PL expression by Western blotting. (D) Cell lysates from control and LPS (100 ng/ml, 3 h)-treated HLMVECs were assayed for S1PL activity using [4,5- 3 H]dihydro S1P as substrate. The product of [4,5- 3 H]dihydro S1P hydrolysis, [3 H]hexadecenal, was separated by thin-layer chromatography, and radioactivity was measured by scintillation counter. Values are means ($\pm$ SE) from three independent experiments in triplicate. Cont, control; Veh, vehicle.

overexpression of S1PL modulates LPS-mediated p38 MAPK and NF- κ B activation in HLMVECs.

S1PL Regulates LPS-Induced Lung Endothelial Barrier Disruption through Rac1

Having shown a regulatory role of S1PL in modulating intracellular S1P levels and LPS-induced IL-6 secretion, we evaluated a potential link between S1PL and barrier dysregulation mediated by LPS. HLMVECs grown on gold electrodes were transfected with scrambled or S1PL siRNA (50 nM, 72 h) and challenged with LPS (100 ng/ml), and TER was measured. As shown in Figure 7A, in scrambled siRNA-treated cells, LPS challenge (100 ng/ml, 6 h) reduced TER, an index of barrier disruption (39); however, down-regulation of S1PL with siRNA partly prevented LPS-induced decrease in TER, suggesting a protective role for intracellular S1P in LPS-induced barrier disruption. To define mechanism(s) of barrier protection by intracellularly generated S1P, we next investigated the role of S1PL and Rac1 in endothelial barrier disruption by LPS. HLMVECs grown on gold electrodes to approximately 70% confluence were infected with adenoviral vector control or dominant negative Rac1 (5 multiplicity of infection) for 24 hours before LPS challenge. LPS treatment increased endothelial permeability,

Figure 5. Overexpression or down-regulation of S1PL modulates LPS-induced IL-6 secretion. (A) HLMVECs (60% confluence) were transfected with S1PL wild-type (WT) plasmid (1 μ g cDNA) in Fugene 6 for 48 hours, and cells were challenged with LPS (100 ng/ml) for 3 hours. Increased S1PL expression by S1PL WT was verified by Western blotting with anti-S1PL antibody. LPS-induced IL-6 secretion was measured by ELISA kit. (B) HLMVECs were transfected with scrambled or S1PL short interfering RNA (siRNA) (50 nM) for 72 hours, and cells were challenged with LPS (100 ng/ml) for 3 hours. Down-regulation of S1PL expression by siRNA, as compared with scrambled siRNA, was verified by Western blotting. LPS-mediated IL-6 secretion was analyzed by ELISA kit. (C and D) HLMVECs (~60% confluence) were transfected with scrambled or S1PL siRNA (50 nM) for 72 hours, pretreated with sodium vanadate (1 mM for 1 h), followed by quantification of S1P levels in medium (C) and in cells (D) by LC-MS/MS. The data represent means ($\pm$ SE) from three independent experiments.

as determined by TER, and overexpression of dominant negative Rac1 potentiated LPS-induced permeability change (Figure 7B). Furthermore, down-regulation of S1PL by S1PL siRNA enhanced Rac1 redistribution to cell periphery (Figure 7C) and Rac1 activity (Figure 7D). Whereas extracellular S1P treatment increased TER in human pulmonary artery ECs (9, 11, 13), exogenous addition of S1P (1 μ M) had little effect on basal as well as LPS-induced changes in TER in HLMVECs (data not shown). These combined results suggest that intracellular accumulation of S1P, compared with exogenous S1P, is more effective in offering protection against LPS-induced endothelial barrier disruption regulated by Rac1 activation and translocation to cell periphery.

S1PL Regulates Rac1 Redistribution to Cell Periphery via S1P₁

Because down-regulation of S1PL with siRNA offered protection against LPS-induced endothelial barrier disruption, we next determined the mechanism involved in intracellularly generated S1P signaling. HLMVECs were pretreated with 4-deoxyypyridoxine (4-DP) (1 mM), which blocks S1PL activity, for 3 hours in the absence or presence of pertussis toxin (PTx; 100 ng/ml) before S1P challenge. As shown in Figures 8A and 8B, PTx significantly blocked S1P- or 4-DP-induced Rac1 redistribution to cell periphery. These results show that redistribution of Rac1 due to S1P accumulation by 4-DP is

Figure 6. S1PL modulates LPS-induced signal transduction. (A) HLMVECs (~60% confluence) were transfected with scrambled or S1PL siRNA (50 nM) for 72 hours. Cells were challenged with LPS (100 ng/ml) for 2 hours. LPS-induced modulation of phosphorylation of p38 and I- κ B, as well as total expression of p38 mitogen-activated protein kinase (MAPK), and β -actin were determined by Western blotting. (B) HLMVECs (~60% confluence) were transfected with scrambled or S1PL siRNA (50 nM) for 72 hours. Cells were challenged with EBM-2 with 1% FBS or Endothelial Cell Basal Medium-2 with 1% FBS containing TNF- α (20 ng/ml) for 10 minutes. TNF- α induced modulation of phosphorylation of p38 and I- κ B, total expression of p38 MAPK, and β -actin were determined by Western blotting. (C) HLMVECs (~60% confluence) were transfected with S1PL WT (1 μ g) plasmid for 48 hours, and then challenged with LPS (100 ng/ml) for 2 hours. Phosphorylation of p38 MAPK, I- κ B, total expression of p38 MAPK, and β -actin were detected by Western blotting with specific antibodies. Shown are representative blots from three independent experiments.

sensitive to PTx, suggesting involvement of Gi-coupled signaling. To determine the role of S1P receptors in this process, we next studied the role of S1P₁ and S1P₃ on basal, S1P, or 4-DP-induced Rac1 redistribution. Stimulation of Rac1 redistribution to cell periphery by S1P or 4-DP was attenuated by S1P₁ siRNA, but not S1P₃ siRNA (Figures E5A–E5B). These results suggest that redistribution of Rac1 by intracellularly generated S1P requires coupling to S1P₁.

DISCUSSION

Acute and subacute inflammatory lung injuries are common, but devastating disorders result from insults, including sepsis, ventilator-induced lung injury, ischemia–reperfusion, hyperoxia, and radiation therapy for thoracic malignancies. Despite recent advances in our understanding of the mechanisms and pathophysiology of ALI (29, 30), specific therapies currently do

Figure 7. Down-regulation of S1PL modulates LPS-induced EC barrier disruption and Rac1 activation. (A) HLMVECs (~60% confluence) grown on gold electrical cell-substrate impedance sensing system (ECIS) electrodes were transfected with scrambled or S1PL siRNA (50 nM) for 72 hours. Cells were challenged with LPS (100 ng/ml) for 6 hours, and changes in transendothelial resistance (TER) were measured by ECIS. (B) HLMVECs (~80% confluence) were infected with adenovirus empty vector or dominant negative Rac1 (Dn-Rac1) adenovirus (10 multiplicity of infection, 24 h) before LPS (100 ng/ml) challenge for 6 hours, and TER was measured by ECIS. The data represent means (+SEM) from three independent experiments. (C) HLMVECs (~60% confluence) grown on glass cover slips were transfected with scrambled or S1PL siRNA (50 nM) for 72 hours, fixed, permeabilized, probed with anti-Rac1 antibody (green), and examined by immunofluorescence microscopy using a 60× oil objective. Cell nucleus was stained with 4′6-diamidino-2-phenylindole (blue). Shown is an immunofluorescence micrograph of three experiments. (D) HLMVECs (~60% confluence) were transfected with scrambled or S1PL siRNA (50 nM) for 72

hours, and then challenged with LPS (100 ng/ml) for 2 hours. Activated Rac1 was immunoprecipitated from total cell lysates (500 µg total protein) of control and LPS-challenged cells using PAK-1 PBD-agarose beads. Rac1-GTP bound to PAK-1 PBD were separated by SDS-PAGE, and probed with anti-Rac1 antibody. Shown is a representative blot from three independent experiments.

not exist for the treatment of ALI. In this study, we have assessed the role of S1PL that regulates intracellular S1P levels as a novel therapeutic target of ALI. The findings of this study show that genetic knockdown of S1PL is associated with enhanced accumulation of S1P in lung tissue as well as BAL fluid, and reduced multiple ALI indices of lung injury. Furthermore, we demonstrate that LPS-mediated IL-6 secretion and endothelial barrier dysfunction was sensitive to intracellular S1P that was regulated by S1PL expression and activity. Our findings also provide new insights into potential mechanism(s) of intracellularly generated S1P in ameliorating LPS-induced lung injury by regulating p38 MAPK, NF-κB, and Rac1 signal transduction.

Alteration in vascular endothelial integrity with increased permeability of fluid and macromolecules into the interstitium and alveolar space are hallmarks of ALI (30, 31). In this regard, we demonstrated that intravenously delivered S1P (11, 16, 17), S1P analogs, or targeted deletion of S1P₃ and S1P₂ receptors as potential therapies for reducing vascular leakage in inflammatory lung injury (38, 39). However, the potential and sustained barrier-enhancing properties of exogenously administered S1P have some limitations due to side effects such as bradycardia and airway hyper-responsiveness (39). Thus, modulation of intracellular S1P production via biosynthesis or degradation offers an attractive alternate approach to protection against LPS-induced vascular alveolar dysfunction in ALI. S1P-mediated cellular responses are regulated by its synthesis, catalyzed by SphKs (2, 3, 6), and degradation mediated by S1P phosphatases (5, 6) and S1PL (4, 7, 8). Genetic or siRNA deletion of SphK1 has been shown to augment LPS-induced lung injury, most likely due to less S1P production in the lung (32, 33); however, mechanism(s) of SphK1 deletion in ALI has not been established. The involvement of SphKs in lung injury is controversial, and depends on the insult. SphK1^{-/-} mice exhibited enhanced pulmonary leak and injury to LPS, whereas adenoviral delivery of SphK1 gene to SphK1^{-/-} mice decreased LPS-mediated vascular leak and inflammation, suggesting a protective role for SphK1 (33). However, reduction of SphK1 expression by siRNA or inhibition of activity decreased allergen-induced airway inflammation in an ovalbumin-sensitized and -challenged murine model

of asthma (40). In contrast to this finding, airway hyperresponsiveness was reduced in the absence of SphK1 in an acute allergen challenge model of inflammation in mice (41, 42). Both *SphK1* and *SphK2* genes are present in mouse and human lung, and, in the absence of SphK1, SphK2 phosphorylates sphingosine to S1P that could address the controversy related to the role of SphK1 and/or SphK2 in lung inflammation.

The present study shows that LPS challenge increases IL-6 secretion and pulmonary leak with a concomitant increase in S1PL protein expression and a decrease in S1P levels in lung tissue of WT mice challenged with LPS. However, S1PL^{+/-} mice developed less pulmonary leak and inflammation that were paralleled by higher S1P levels in lung tissue and BAL fluid. Treatment with THI, a component of food colorant, increases S1P levels in lung tissue and ameliorates LPS-induced lung inflammation and injury, confirming a potential role for S1PL in ALI. THI blocks S1PL activity when administered orally, and S1PL inhibition has been demonstrated with a regimen of 3 days of treatment of THI (27). In our experiments with THI, tissue S1P levels were elevated by Day 2 of treatment without altering plasma S1P levels. Furthermore, prolonged treatment would not be advisable, considering the long-term effects of S1PL knockout in genetic models, which have very high plasma and tissue S1P levels (25), which are confounded by disruption of lipid homeostasis and accumulation of ceramides and other sphingolipids in liver (43). Inhibition of S1PL by THI in animals correlates with reduced lyase activity in lymphoid tissue; however, mechanism of this inhibition remains unclear.

Our present study does not address which cells in the lung have increased S1PL expression and which ones are responsible for increased S1P levels. However, there are at least five major cell types present in the lung that might exhibit increased S1PL expression in response to LPS under *in vivo* conditions. Furthermore, several immune-modulator cells, such as lymphocytes, neutrophils, macrophages, and eosinophils infiltrate into the alveolar space that makes it extremely difficult to determine the source of S1P. In addition to LPS, S1PL expression has been shown to be regulated by other stress conditions, such as DNA damage (24), vascular shear stress (44), GATA transcription factors (45), and as an early response gene regulated by PDGF (46).

Figure 8. Pertussis toxin (PTx) attenuates 4-deoxyphalloidin (4-DP)- or S1P-induced translocation of Rac1 to cell periphery in HLMVECs. (A) HLMVECs grown on glass cover slips (~90% confluence) were pre-treated with PTx (100 ng/ml) for 4 hours before exposure to 4-DP (1 mM) or S1P (1 μ M) for an additional 2 hours. Cells were washed, fixed, permeabilized, and probed with anti-Rac1 antibody. A representative immunofluorescence micrograph from three independent experiments is shown. (B) Quantification of Rac1 translocation was performed using image analysis software, and values are the mean ($\pm$ SE) from three independent experiments.

Potential modulation of S1P receptors by high levels of alveolar or circulating S1P may also contribute to impaired lung function of S1PL^{-/-} mice. Presently, no information is available on the expression levels of S1P receptors in S1PL^{-/-} mice compared with S1P^{+/-} and WT animals. However, it is important to point out that potential therapeutic effect of exogenous S1P in lung injury is dependent on the route of delivery, dose, and relative expression of S1P receptors. Although intravenous administration of S1P- or S1P₁-selective agonist (SEW-2871) produced a dose-dependent (0.3 mg/kg) alveolar and vascular barrier protection against LPS-mediated lung injury, intratracheal delivery of S1P (0.5 mg/kg) resulted in alveolar barrier disruption in mice (39). Furthermore, S1P₁^{+/-} heterozygous mice were partially refractory to protective effects of S1P, whereas mice deficient in S1P2 and S1P3 were less susceptible to LPS-induced lung injury (39).

Results presented here demonstrate that increased S1P levels in lung ECs due to S1PL siRNA mitigates LPS-induced signal transduction, IL-6 secretion, and barrier dysfunction. Furthermore, exogenous addition of hexadecanal or ethanolamine phosphate had no effect on LPS-induced p38 MAPK activation or I-kB phosphorylation in HLMVECs (Figure E6). However, in mouse F9 embryonic carcinoma cell lines, products of S1PL pathway (hexadecanal and ethanolamine phosphate), but not intracellular accumulation of S1P, stimulated mitogenesis (47). Thus, although we cannot rule out a biological role for S1PL products in other systems (47), in human lung endothelium it is the intracellularly generated S1P, but not S1PL products, that regulate LPS signaling and secretion of IL-6.

Although much is known about the extracellular action of S1P via specific G protein-coupled S1P receptors, little is known as to the biological actions and targets of intracellularly generated S1P (18). It is becoming apparent that intracellularly produced S1P can signal either intracellularly or extracellularly after secretion of the S1P generated inside the cell mediated by members of the ATP-binding cassette family of transporters, such as ABCB1 (48, 49), and by the recently identified S1P transporter, Spns2 (50). Our studies on the measurement of S1P in cells and media by LC-MS/MS show very little accumulation of S1P in media as compared with cells under the experimental conditions; however, addition of sodium orthovanadate, a general inhibitor of protein tyrosine phosphatases (34), enhanced S1P levels in medium as well as inside the cells, suggesting secretion of S1P and possible catabolism of the secreted S1P by LPPs on the cell surface. In fact, overexpression of LPP-1 enhances intracellular accumulation of [³²P]S1P, and knockdown of LPP-1 decreases intracellular S1P from exogenous S1P in lung ECs (19). Furthermore, down-regulation of LPP-1 by LPP-1 siRNA decreases LPS-mediated endothelial barrier dysfunction (data not shown) that might involve modulation of S1P accumulation in milieu. In this study, we have demonstrated that redistribution of Rac1 by intracellularly generated S1P requires coupling to S1P₁. Under normal conditions of incubation, we could not detect accumulation of S1P in the medium with scrambled or S1PL siRNA treatment of cells (data not shown); however, addition of sodium vanadate to the incubation significantly enhanced S1P in the medium as well as in cells (Figures 5C and 5D). These results suggest that HLMVECs have the ability to secrete intracellularly generated S1P, which gets degraded rapidly most likely by LPPs located on the cell surface (19). Interestingly, accumulation of S1P was detectable by inhibiting the LPPs with sodium vanadate. A similar accumulation of S1P in the medium was observed in cells treated with LPP-1 siRNA (data not shown).

Earlier reports suggested platelets to be a source of plasma S1P (51, 52); however, recent studies suggest that red blood cells and vascular endothelium, but not platelets, may serve as major sources of plasma S1P (44, 53, 54). Another S1PL inhibitor, LX2931 (Lexicon Pharmaceuticals, Woodlands, TX), is currently in phase 1 clinical trials for rheumatoid arthritis; thus, S1PL inhibitor drugs developed for immunomodulation purposes may prove useful in clinical settings against ALI and inflammation. Thus, our results also suggest the ability of pulmonary endothelium to increase intracellularly formed S1P resulting from blocking S1PL, and a potential role for this S1P in protection against LPS-induced lung injury.

The present findings on the balance between intracellular S1P production and involvement of signaling via S1P₁ as a potential mechanism of action of S1PL blocking in protection against LPS-induced lung injury raises the question of potential mechanism(s) of S1P release from the endothelium. Involvement of ATP binding cassettes, ABCB1, ABCA1, ABCG2 (48–

50, 55), or Spns2 (50), in S1P release has been demonstrated in other cell types, but not from the endothelium. Second, the effect of combined blocking of S1PL and LPPs/S1P phosphatases on S1P release may be therapeutically beneficial, which warrants additional investigation.

In summary, our results show that LPS decreases intracellular S1P levels via up-regulation of S1PL expression in mouse lung and human lung microvascular ECs. Furthermore, our results highlight the importance of intracellular accumulation of S1P, by blocking S1PL expression or activity, in ameliorating LPS-induced inflammation and barrier dysfunction in murine and cell culture models of ALI. Collectively, these findings link S1P biosynthesis and signaling to ALI and S1PL as a potential target for S1P-based therapies for inflammatory lung injury.

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