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Epigenetic regulation of pro-inflammatory cytokine secretion by sphingosine 1-phosphate (S1P) in acute lung injury: Role of S1P Lyase

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Abstract

Cellular level of sphingosine-1-phosphate (S1P), the simplest bioactive sphingolipid, is tightly regulated by its synthesis catalyzed by sphingosine kinases (SphKs) 1 & 2 and degradation mediated by S1P phosphatases, lipid phosphate phosphatases, and S1P lyase. The pleiotropic actions of S1P are attributed to its unique inside-out (extracellular) signaling via G-protein-coupled S1P1-5 receptors, and intracellular receptor independent signaling. Additionally, S1P generated in the nucleus by nuclear SphK2 modulates HDAC1/2 activity, regulates histone acetylation, and transcription of pro-inflammatory genes. Here, we present data on the role of S1P lyase mediated S1P signaling in regulating LPS-induced inflammation in lung endothelium. Blocking S1P lyase expression or activity attenuated LPS-induced histone acetylation and secretion of pro-inflammatory cytokines. Degradation of S1P by S1P lyase generates 2-hexadecenal and ethanolamine phosphate and the long-chain fatty aldehyde produced in the cytoplasmic compartment of the endothelial cell seems to modulate histone acetylation pattern, which is different from the nuclear SphK2/S1P signaling and inhibition of HDAC1/2. These *in vitro* studies suggest that S1P derived long-chain fatty aldehyde may be an epigenetic regulator of pro-inflammatory genes in sepsis-induced lung inflammation. Trapping fatty aldehydes and other short chain aldehydes such as 4-hydroxynonenal derived from S1P degradation and lipid peroxidation, respectively by cell permeable agents such as phloretin or other aldehyde trapping

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Competing Interests Statement

The authors have no competing financial interests.

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agents may be useful in treating sepsis-induced lung inflammation via modulation of histone acetylation.

Keywords

Acute Lung Injury; S1P signaling; Histone Acetylation; S1P Lyase; Sphingosine Kinases; Hexadecenal; Histone Deacetylases

INTRODUCTION

Acute lung injury (ALI) (sepsis, ventilator-induced lung injury, ischemia/reperfusion, hyperoxia) and the subacute inflammatory lung injury, which occurs following thoracic exposure to ionizing radiation, a syndrome known as radiation-induced lung injury (RILI) share common signaling pathways and increased vascular permeability as key elements, resulting in increased morbidity and mortality (>35%). Despite advances in better understanding of lung pathophysiology, specific therapies currently do not exist for the treatment of ALI or for alleviation of the unremitting vascular leak, which serves as a defining feature of these illnesses. For example, high dose and long term corticosteroids remain the sole treatment for RILI but have limited utility due to relative ineffectiveness and severe side effects. Therefore, there is a need for new mechanistic insights that can lead to novel strategies, biomarkers, and therapies to reduce the morbidity and mortality of these acute and sub-acute inflammatory injuries.

In the last decade, sphingolipid mediators including sphingosine, ceramide and sphingosine-1-phosphate (S1P), S1P receptors (S1PRs), and enzymes of S1P metabolism, have been identified as key modulators of several human pathologies including pulmonary diseases (Natarajan et al., 2013; Ebenezer et al., 2016a), cancers (Pyne and Pyne, 2010; Pyne et al., 2014) including breast cancer (Macziz et al., 2016) and immune responses (Spiegel and Milstien, 2011; Hisano et al., 2012). Among the sphingolipid metabolites, S1P has been shown to have both beneficial and detrimental effect on lung inflammation and vascular integrity. S1P is enriched in plasma compared to tissues and interstitial fluids (Olivera et al., 2013). Cellular sources of S1P in blood are unclear however, as recent studies suggest that platelets (Yatomi et al., 2004), red cells (Ito et al., 2007), and non-hematopoietic sources such as liver sinusoidal endothelial cells (ECs) (Venkataraman et al., 2008) may be predominant sources of plasma S1P (Ksiek et al., 2015). Most mammalian cells synthesize S1P by phosphorylation of sphingosine, catalyzed by sphingosine kinases (SphKs) 1 & 2. Recent studies have shown that ecto-lipid phosphate phosphatases (LPPs) regulate intracellular generation of S1P from exogenous S1P. LPP1-catalyzed dephosphorylation of S1P to sphingosine and subsequent uptake of the resulting sphingosine, and re-phosphorylation by SphK1 to S1P in lung ECs (Zhao et al., 2007), suggesting the ability of endothelial and other cell types to generate intracellular S1P from plasma to act in a autocrine/paracrine fashion to stimulate S1PRs present at the cell surface (“inside-out” signaling) and initiate downstream G-protein-coupled receptor signaling and cellular responses. S1P, generated within cells is metabolized back to sphingosine by S1P phosphatases (SPPs) 1 & 2. Additionally, S1P is also terminally hydrolyzed by S1P lyase

(S1PL) to ethanolamine phosphate and hexadecenal in cells (Van Veldhoven, 2000), (Saba and Hla, 2004) Thus, S1P accumulation in cells is regulated between synthesis, catalyzed by SphKs, and degradation mediated by LPPs, SPPs, and S1PL. S1P action may be dependent on the activation and spatial distribution of its metabolizing enzymes as well as the availability of sphingosine, the substrate. Thus, modulation of SphKs, SPPs/LPPs, and S1PL may affect LPS-induced inflammatory signaling, cytokine production, and lung vascular barrier function. In addition to inside-out signaling, S1P can also signal inside the cell by increasing free Ca^{2+} levels (Birchwood et al., 2001; Itagaki and Hauser, 2003); however, the intracellular target(s) is yet to be defined. Recent studies suggest binding of S1P to human telomerase (Panneer Selvam et al., 2015) and HDACs 1& 2 in cancer cells (Hait et al., 2009), which warrants more detailed investigation in terms of physiology and pathophysiology. Here, we have identified a role for S1P as an epigenetic co-regulator in a murine model of LPS-induced ALI.

Sphingosine Kinases, S1P Lyase, and Intracellular S1P in LPS-induced acute lung injury

A protective role of S1P in LPS-induced lung injury is well established. Intravenously administered S1P or S1P analogs reduced vascular leak and pulmonary edema in murine and canine models of sepsis-induced lung injury (McVerry and Garcia, 2004). Intratracheal administration of LPS stimulated expression of S1PL and decreased S1P levels in mouse lung as well as in human lung microvascular ECs (Fig. 1) (Zhao et al., 2011). Accumulation of intracellular S1P is a balance between its formation catalyzed by SphK1 and SphK2 and catabolism mediated by SPPs, LPPs and S1PL. Genetic deletion of SphK1 enhanced pulmonary leak and inflammation, while deletion of SphK2 in mice had no significant effect on LPS-induced lung inflammation, suggesting a protective role for SphK1/S1P signaling (Wadgaonkar et al., 2009). Decreased S1P levels in lung tissues after intratracheal LPS administration could be in part due to enhanced S1PL expression/activity (Fig. 1) and/or decreased SphK expression/activity. Blocking S1PL activity with 2-acetyl-4(5)-[1(R),2(S),3(R),4-tetrahydroxybutyl]-imidazole (THI) or partial deletion of *Sgpl1* resulted in increased S1P levels in mouse lung tissue, BAL fluids, and reduced LPS-induced lung inflammation and injury (Zhao et al., 2011). Down-regulation of S1PL expression attenuated LPS-induced pro-inflammatory signals including phosphorylation of I- κ B and p38 MAPK without affecting the expression levels of total p38 MAPK, while over-expression of S1PL WT to HLMVECs potentiated LPS-mediated phosphorylation of I- κ B and p38 MAPK (Fig. 2). Further, blocking NF- κ B activation by Bay11-7082 attenuated LPS-induced IL-6 secretion (Fig 2), suggesting a novel role for NF- κ B signaling and intracellularly accumulating S1P in reducing inflammatory signaling pathways of IL-6 production. Conversely, activation of SphK1 with K6PC-5 [N-(1,3-dihydroxyisopropyl)-2-hexyl-3-oxo-decanamide] or Unicamide-AA () resulted in increased S1P levels in HLMVECs and decreased LPS-induced phosphorylation of p38 MAPK, I- κ B, JNK (Fig. 3 A & B), and as well as IL-6 release (Fig. 3C). These results suggest that altering S1P levels in cells either by down-regulation of S1PL or activation of SphK modulate LPS-mediated pro-inflammatory signals and IL-6 release.

Emerging role of S1P signaling and chromatin modification

Epigenetic events like histone acetylation can activate transcription by rendering gene promoters more accessible to the transcription machinery. Histone acetylation is regulated by histone acetyltransferases (HATs) and histone deacetylases (HDACs). The activities of HATs and HDAC are tightly controlled through targeted recruitment, specific protein-protein interactions, and post-translational modifications, such as phosphorylation (Bannister and Kouzarides, 2011; Delcuve et al., 2012; Hassig and Schreiber, 1997). In vascular smooth muscle cells, angiotensin II-induced IL-6 expression is known to require activation of NF- κ B and CREB as well as ERK-dependent histone acetylation mediated p300 and SRC-1 (Sahar et al., 2007). However, in breast cancer cells, IL-8 expression is regulated by HDACs through NF- κ B pathway (Chavey et al., 2008). Very little is known regarding endogenous S1P as regulators of HATs/HDACs. Recent reports provide evidence for nuclear S1P or FTY720-P binding to HDAC1 and HDAC2 and inhibiting its activity (Hait et al., 2015). Binding of S1P to HDAC1/2 in the nucleus requires spatio-temporal generation of nuclear S1P by localized SphK1 or SphK2. Current evidence points out to nuclear localization of SphK2 and not SphK1 in PMA-induced S1P production in the nucleus. In contrast to SphK1, nuclear import as well export sequences have been identified in mammalian SphK2 (Ding et al., 2007; Igarashi et al., 2003).

LPS stimulates histone acetylation in mouse lung tissue and human lung endothelial cells

Intratracheal instillation of LPS to C57BL/6 wild type mice stimulated acetylation of H3 and H4 histones in mouse lung tissue as well as human lung microvascular ECs exposed to LPS (Fig 4A & 4 B). Genetic knockdown of SphK1, but not SphK2, attenuated LPS-induced histone acetylation (Fig. 4C), suggesting a role for SphK1/S1P signaling in histone acetylation and chromatin modification. However, *Pseudomonas aeruginosa* infection of mouse lung stimulated Histone H3 & H4 acetylation and phosphorylation of SphK2, but not SphK1 (Fu et al., 2014). LPS enhanced global acetylation of histones in HLMVECs, and as increased transcription is linked to chromatin reorganization mediated by acetylation of histones, we examined the role of HDACs and HATs in IL-6 release from human lung ECs by LPS. Treatment of HLMVECs with trichostatin A (TSA) (1 μ M for 3 h), an inhibitor of HDACs, increased basal and LPS-mediated IL-6 release (Fig. 5A); however, inhibition of HAT with a HAT inhibitor, anacardic acid (10 μ M), attenuated LPS-induced IL-6 (Fig. 5B). These results suggest that the LPS-induced IL-6 secretion in the endothelium is regulated by HDACs/HATs.

Down-regulation or inhibition of S1PL attenuates LPS-induced histone acetylation and IL-6 secretion

S1PL is a pyridoxal phosphate-dependent enzyme that irreversibly cleaves S1P to ethanolamine phosphate and 2-hexadecenal, which is oxidized to *trans-2-hexadecenoic acid or alcohol* and recycled into glycerophospholipids or sphingolipids in mammalian cells (Saba and Hla, 2004). S1PL is mainly localized in the endoplasmic reticulum of cells and

there is growing evidence for its role in lung pathologies such as sepsis (Zhao et al., 2011), pulmonary fibrosis (Huang and Natarajan, 2015), and bronchopulmonary dysplasia (Harijith et al., 2013). Inhibition of S1PL offers protection against LPS-induced murine acute lung injury (Zhao et al., 2011) and hyperoxia-induced lung inflammation (Harijith et al., 2013) by enhancing S1P levels in lung tissue. S1PL has been shown to be an endogenous suppressor of pulmonary fibrosis (Huang et al., 2015a; Huang et al., 2015b). S1PL expression was enhanced in fibrotic lung tissues and primary lung fibroblasts isolated from IPF patients and bleomycin challenged mice. TGF- β increased expression of S1PL in human lung fibroblasts via activation of Smad3 signal transduction and over-expression of S1PL attenuated TGF- β mediated differentiation of human lung fibroblasts to myofibroblasts through regulation of LC3 and beclin 1, markers of autophagy (Huang et al., 2015b). Knockdown of S1PL in mice augmented bleomycin-induced pulmonary fibrosis, and idiopathic pulmonary fibrosis patients with reduced *Sgpl1* mRNA expression in PBMCs exhibited higher severity of fibrosis and lower survival rate (Huang et al., 2015b). There is also evidence for a link between histone deacetylation and Ca^{2+} homeostasis in S1PL deficient mouse embryonic fibroblasts (Ihlefeld et al., 2012). As S1PL regulates intracellular S1P levels in cells, inhibition of S1PL results in increased S1P levels in cells including the nucleus (Ihlefeld et al., 2012), which may have an effect on binding to histones and HDAC activity. Inhibition of S1PL activity by 4-deoxypyridoxine (Schwab et al., 2005) attenuated LPS-induced acetylation of H3 and H4 histones, while activation of SphK1 with an activator K6PC-5 (Hong et al., 2008; Park et al., 2008) also resulted in reduced histone acetylation after LPS challenge of human lung ECs (Fig. 6). Inhibition of S1PL activity with 4-deoxypyridoxine attenuated LPS-induced IL-6 secretion (data not shown), and over-expression of S1PL WT in lung ECs potentiated LPS-induced IL-6 secretion ((Zhao et al., 2011). In complimentary studies, overexpression of S1PL potentiated LPS-induced H3 & H4 histone acetylation (data not shown). These results suggest that increasing intracellular S1P levels either by blocking S1PL or activation of SphK modulates histone acetylation pattern and IL-6 secretion in lung ECs. Interestingly, an increase in basal H3/H4 histone acetylation was observed in cells treated with 4-deoxypyridoxine (Fig. 6), suggesting increased nuclear S1P level may be involved in enhancing basal histone acetylation levels, as suggested earlier (Ihlefeld et al., 2012).

Knockdown of S1PL modulates promoter activity of *IL-6*, *HAT1*, and *MYST2* and expression of HDACs

Next, we examined the effect of S1PL siRNA on activity of promoter regions using chromatin immuno-precipitation (ChIP) with acetyl lysine antibody. ChIP DNA was subjected to PCR amplification for *IL-6*, *HAT1*, and *MYST2* genes, which are involved in the process of inflammation. As shown in Fig. 7A, treatment of scrambled siRNA transfected cells with LPS increased the promoter activity of *IL-6*, which was reduced by S1PL siRNA. Further, S1PL siRNA decreased *MYST2* promoter activity induced by LPS. Analysis of the protein expression revealed enhanced HDAC5 and decreased HDAC10 expressions in S1PL down-regulated cells (Fig. 7B). Thus, the present study suggests that down-regulation of S1PL (increase of S1P levels) not only affects histone acetylation pattern, but may also modulate expression of class II and class III HDACs by an unknown

mechanism. HDACs can be regulated by phosphorylation, acetylation, nitrosylation, sumoylation or ubiquitination, and subsequent degradation. In S1PL deficient mouse embryonic fibroblasts, a decrease in HDAC1 and HDAC3 expression was observed (Ihlefeld et al., 2012); however, mechanism(s) of reduced expression is unclear. How knockdown of S1PL increases nuclear S1P levels is unknown; however, S1P generated in the nucleus by SphK2 or transported from the cytoplasmic compartment may be functionally different in modulating HDAC activity or expression.

Association of nuclear SphKs with the histones, HDACs and corepressor complex

Sphingolipids have been shown to be associated with chromatin with sphingosine as a major component of the chromatin associated sphingolipid (Lucki and Sewer, 2012). Recent studies suggest a direct binding of S1P to HDAC1/HDAC2 in cancer cells (Hait et al., 2009), and human telomerase (hTERT) (Panneer Selvam et al., 2015), and S1P generated by nuclear SphK2 as an inhibitor of HDAC1/2 activity in cancer cells (Hait et al., 2009). Further, both SphK2 or phosphorylated SphK2 and S1P were identified as part of the corepressor complex associated with HDACs1/2 and promoters of transcriptionally silent genes (Hait et al., 2009). HDACs1/2 associated with the corepressor(s) contributes to gene repression by deacetylating lysine residues of histones. Sphingosine and dihydrosphingosine were detected in HDAC1 immunoprecipitates from nuclei of mouse lung epithelial cells (MLE-12) and *Pseudomonas aeruginosa* treatment reduced the sphingosine levels (Fig. 8A). Further, we were able to demonstrate binding of S1P to purified HDAC1/2 *in vitro* (Fig. 8B); however, the S1P binding to HDAC1/2 failed to inhibit HDAC activity (data not shown), suggesting binding alone may be insufficient to modulate HDAC activity (Hait et al., 2009). Nitric oxide (NO) has been shown to inhibit HDAC2 association with chromatin by nitrosylation of two cysteine residues located outside the catalytic domain, thereby not affecting its activity (Lindermayr, 2013). The mechanism of HDAC1/2 inhibition by S1P generated after PMA treatment of cancer cells (Hait et al., 2009) is unclear but probably involves binding to the active site occupied by suberoylanilide hydroxamic acid, a potent and selective class I and II HDAC inhibitor. Instillation of LPS to mouse lung or exposure of ECs to LPS enhanced expression of S1PL and decreased S1P levels in lung tissues post-LPS challenge (Zhao et al., 2011). The decrease in S1P could be attributed to enhanced S1PL expression after LPS challenge since LPS did not stimulate SphK1 or phosphorylation (data not shown). This is in contrast to *P. aeruginosa* infection of mouse lung that increased global acetylation of histones and genetic deletion of SphK2, but not SphK1, mitigated PA-mediated histone acetylation and generation of IL-6, and TNF- α production both in mouse lungs and mouse lung epithelial cells (Fu et al., 2014; Ebenezer et al., 2016b).

Mechanism(s) of LPS-mediated enhanced nuclear histone H3/H4 acetylation by cytoplasmic S1P or S1P metabolite 2-hexadecenal

There is recent evidence for S1P generated in the nucleus by SphK2 to bind to HDACs and inhibit HDAC1/2 activity either by direct binding of S1P to HDACs1/2 (Hait et al., 2009) or oxidation of SH groups in HDAC 4 by ROS generated via nuclear Nox4 (Matsushima et al.,

2013); however, mechanism(s) of HDAC1/2 inhibition or increased histone acetylation by cytosolic SphK1/S1P signaling axis is unknown. S1PL catalyzes the hydrolysis of S1P to 2-hexadecenal and ethanolamine phosphate (Saba and Hla, 2004). 2-Hexadecenal is a reactive aldehyde that could form adducts with primary amino groups of lysine or N-terminal residues similar to other short chain aldehydes such as acrolein (cigarette smoke component) or 4-hydroxynonenal (lipid peroxidation product). Thus, formation of protein adducts, and Michael addition products by binding of aldehydes on amino groups of lysine or histidine or thiols (Cysteine or GSH), can modulate normal cellular functions. As LPS challenge enhanced S1PL expression *in vivo* and *in vitro*, we hypothesized that this resulted in higher S1P degradation and the generation of 2-hexadecenal in the cytoplasm/endoplasmic compartment of the cell, which can modify HDACs1/2 or HATs to alter histone acetylation. Blocking S1PL with siRNA or inhibition of S1PL with inhibitors such as 4-deoxypyridoxine or Novartis compound 32 attenuated LPS-mediated histone acetylation in human lung ECs (Fig. 6). Interestingly, trapping of long chain aldehydes with phloretin (Ullen et al., 2012) also reduced LPS-induced H3/H4 histone acetylation (Fig 9). Further, exogenous addition of 2-hexadecenal to nuclear preparations from MLE-12 cells stimulated H3/H4 histone acetylation (data not shown), indicating a role for hexadecenal released from S1P could be a modulator of histone acetylation and inflammation. However, we cannot rule out the role of LPS-induced ROS generation in lung ECs in lung inflammation and histone modification. LPS stimulates ROS production in lung ECs (MittalManish et al., 2014) and increased ROS may induce lipid peroxidation and release of active aldehydes such as 4-hydroxynonenal, a known modifier of nuclear histones (Drake et al., 2004), and inflammation. In addition to trapping aldehydes, phloretin being a polyphenol may affect ROS levels as an anti-oxidant, which needs to be verified in LPS-induced lung inflammation (Fig. 10). Further studies are necessary to establish and define the role 4-hydroxynonenal generated from lipid peroxidation and long-chain fatty aldehydes such as 2-hexadecenal released from S1P as a potential epigenetic co-regulator of pro-inflammatory genes in lung pathologies.

Conclusions

S1P is a double-edged sword that exhibits both pro-and anti-inflammatory properties depending upon the cell type, site of synthesis and degradation, and pathophysiology of the disease. Although many of the cellular actions of S1P have been attributed to its ligation to G-protein-coupled S1P1-5 receptors on the cell surface, S1P can also signal intracellularly by direct action on intracellular targets such as E3 ubiquitin ligase TRAF-2 (Alvarez et al., 2010), Rac1 (Ryu et al., 2002), β -site amyloid precursor protein cleaving enzyme-1 (Takasugi et al., 2011), telomerase (Panneer Selvam et al., 2015), and Ca^{2+} homeostasis (Birchwood et al., 2001; Itagaki and Hauser, 2003; Ihlefeld et al., 2012). There is emerging evidence demonstrating the ability of intracellularly generated S1P, especially in the nucleus by SphK2, to inhibit HDACs1/2 and modulate histone acetylation and inflammation; Nguyen-Tran et al., 2014). S1P generated in the cytoplasmic compartment also functions as epigenetic co-regulator in LPS-induced lung inflammation involving 2-hexadecenal released from S1P by S1PL. Additionally, S1P can also stimulate ROS production in cells via Nox2 or Nox4 (Ebenezer et al., 2016; Fu et al., 2014), and this ROS can induce lipid

peroxidation resulting in generation of bioactive aldehydes such as 4-hydroxynonenal that can modulate histones via adduct formation or modification of cysteine residues on HDACs (Fig. 10). Thus, inhibition of S1PL or activation of SphK1 or SphK2 would be a novel therapeutic approach to modulate intracellular S1P levels and chromatin assembly via histone acetylation. Further studies are necessary to understand the ability of S1P and its metabolites to regulate HATs and HDACs, and post-translational modifications of histones including acetylation and methylation and thereby functioning as epigenetic co-regulators of lung inflammation, injury, and repair.

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Figure 1. LPS regulates S1PL expression, S1P levels and luciferase reporter activity in HLMVECs

HLMVECs (~90% confluent) were exposed to medium containing 1% FBS or LPS (100 ng/ml) for 3 h. (A) Intracellular S1P levels were quantified by LC/MS/MS. Values are means $\pm$ S.E. from three independent experiments in triplicate, and S1P levels were normalized to total lipid phosphorus. (B) Total RNA was isolated and S1PL mRNA levels were quantified by real time RT-PCR. Values are means $\pm$ S.E. and normalized to 18S rRNA. (C) HLMVECs grown on 35-mm dishes (~70% confluence) were transfected with pGL3 vector and *S1pl*-pGL3-luciferase reporter plasmid (1 μ g/ml) for 24 h prior to LPS (100 ng/ml) challenge for 3 h. Cell lysates were analyzed for luciferase activity using Promega Dual Luciferase activity kit. The values are means $\pm$ S.E. of three independent experiments in triplicate and normalized to *Renilla* internal control luminescence. (D) Cell lysates from control and LPS (100 ng/ml), treated HLMVECs were assayed for S1PL expression at various time points. Shown is a representative Western blot from three independent experiments. Values are mean $\pm$ S.E. from three independent experiments in triplicate and expressed as pmol product formed/min/mg protein. The data represent means $\pm$ S.E. from three independent experiments.

Figure 2. S1PL modulates LPS-induced NF-κB activation, NF-κB mediated IL-6 secretion and p-38 MAPK phosphorylation

(A), HLMVECs (~90% confluence) grown on slide chambers were stimulated with LPS (100 ng/ml) for varying time periods (15, 60 and 120 min), cells were fixed and immunostained with anti-p65 antibody and NF-κB translocation to the nucleus was visualized by immunofluorescence microscope. Shown is a representative micrograph from three independent experiments. LPS enhanced p65 localization in the nucleus at 0 and 120 min post LPS challenge. (B), HLMVECs (~90% confluence) grown on 35-mm dishes were pre-treated with Bay 11-7082 (10 μM) for 60 min prior to LPS (100 ng/ml) challenge for 3 h. The supernatants were collected, centrifuged at 500xg for 10 min and IL-6 levels were measured by a commercial ELISA kit. Values are means ± S.E. from three independent experiments. (C), HLMVECs (~60% confluence) were transfected with scrambled or S1PL siRNA (50 nM) for 72h. Cells were challenged with LPS (100 ng/ml) for 2 h. LPS-induced modulation of phosphorylation of p38 and I-κB as well as total expression of p38 MAPK, and β-actin were determined by Western blotting. (D), HLMVECs (~60% confluence) were transfected with S1PL wild type (1 μg) plasmid for 48 h, and then challenged with LPS (100 ng/ml) for 2 h. Phosphorylation of p38 MAPK, I-κB and total expression of p38 MAPK, and β-actin were detected by Western blotting with specific antibodies. Shown are representative blots from three independent experiments.

Figure 3. SphK activator, K6PC-5, increases intracellular S1P levels and attenuates LPS-induced signaling and IL-6 secretion

(A), HLMVECs (~90% confluence) grown on 35-mm dishes were incubated with SphK activators with K6PC-5 or U-AA (5 and 20 μ M), respectively for 60 min in the absence or presence of exogenous sphingosine (1 μ M), and intracellular S1P levels were quantified by LC/MS/MS and normalized to total lipid phosphorus. Values are means $\pm$ S.E. **, significantly different from cells not pre-treated with K6PC-5. (B), HLMVECs (~90% confluence) were treated with K6PC-5 (20 μ M, 30 min) prior to LPS challenge for 2 h. Cell lysates were analyzed for phosphorylation of p38 MAPK, I- κ B, and JNK by Western blotting. **Panel C.** Media from (B) were collected and IL-6 levels were measured by ELISA kit. Values are means $\pm$ S.E. of three independent experiments.

Figure 4. LPS stimulates H3 and H4 histone acetylation on mouse lung and human lung endothelial cells

(A), LPS (5 mg/Kg body weight) in sterile PBS or PBS alone was administered intratracheally to 8- to 10-week old C57BL/6 wild-type, *SphK1*^{-/-} or *SphK2*^{-/-} mice. After 24 h, mice were anesthetized with 3–5 ml/kg of a mixture of 25 mg/kg of ketamine and 2.5 ml of xylazine, and sacrificed. Lungs were lavaged and perfused and total lung lysates were subjected to SDS-PAGE (30–40 µg of protein) and Western blotting with anti-histone H3K9, anti-histone H4K8, anti-histone H3 or anti-histone H4 antibodies. Shown is a representative blot from three independent experiments. LPS enhanced acetylation of H3 and H4 histones, and genetic knockdown of SphK1 had no effect on the LPS-induced histone acetylation. However, knockdown of SphK2 in mice attenuated H3 and H4 histone acetylation in mouse lung. (B), HLMVECs (~95% confluence) were challenged with LPS (1 µg/ml) for varying time periods (15–180 min) and total cell lysates were subjected to 5–20% SDS-PAGE and Western blotting with anti-histone H3K9, anti-histoneH4K8 and anti-histone H3 antibodies. Shown is a representative Western blot from three independent experiments. LPS stimulated acetylation of histone H3K9 and H4K8 in human lung ECs.

Figure 5. Role of histone deacetylase (HDAC) and histone acetyl transferase (HAT) on LPS-induced IL-6 secretion

HLMVECs (~95% confluence) grown on 35-mm dishes were pre-treated with HDAC inhibitor Trichostin A (TSA) (1 μM) (A) or HAT inhibitor, anachordic acid (AA) (10 μM) (B) for 1 h prior to stimulation with LPS (100 ng/ml) for 3 h. The media was collected and IL-6 levels were measured using an ELISA kit. Values are means ± S.E. of three experiments in triplicate. TSA potentiated LPS-induced IL-6 secretion while AA inhibited the IL-6 accumulation in HLMVECs.

Figure 6. Modulation of LPS-mediated histone acetylation by inhibition of S1PL or activation of SphK

HLMVECs (~90% confluence) grown on 35-mm dishes were pre-treated with S1PL inhibitor, 4-deoxypyridoxine (1 mM) or SphK activator, K6PC-5 (50 μ M) for 18 h prior to exposure to LPS for 1 and 3 h. Cell lysates were subjected to 5–20% SDS-PAGE and Western blotted with anti-acetylated histone H3K9, anti-acetylated histone H4K8, anti-histone H3 and anti-histone H4 antibodies. Shown is a representative blot from three independent experiments.

Figure 7. S1PL modulates LPS induced promoter activity and HDAC5 expression

(**A**), HLMVECs (~70% confluence) grown on 100-mm dishes were transfected with scrambled or S1PL siRNA (100 nM) for 72 h and cells were challenged with LPS (100 ng/ml, 3 h), subjected to immunoprecipitation with ChIP grade NF- κ B antibody and the immunoprecipitates were subjected to RT-PCR for IL-6, HDAC10, HAT1 and MYST2. LPS-induced enhanced binding of NF- κ B to the promoter regions of IL-6, HAT1 and MYST2 that was attenuated by S1PL siRNA in HLMVECs. (**B**), HLMVECs (~60% confluence) grown in 35-mm dishes were transfected with scrambled or s1PL siRNA (100 nM) for 72 h prior to LPS (100 ng/ml) for 3 h. Cell lysates were analyzed by SDS-PAGE and Western blotting with anti-HDAC1, anti-HDAC5, anti-HDAC10, and anti- β -actin antibodies. Shown is a representative blot from three independent experiments.

Figure 8. Association of sphingosine with HDAC1 in the nucleus and binding of S1P to HDAC1 *in vitro*

(A), Mouse alveolar type II epithelial (MLE-12) cells were challenged with vehicle or heat killed *Pseudomonas aeruginosa* lysates (1×10^8 CFU/ml) for 24 h and nuclei were isolated by differential sucrose density gradient centrifugation. Nuclear lysates were subjected to immunoprecipitation with HDAC1 antibody total lipids from HDAC1 immunoprecipitates were isolated and analyzed for sphingosine, dihydro-sphingosine and S1P levels by LC-MS/MS. (B), Biotinylated S1P (100 nM) or sphingosine (100 nM) was added to nuclei (100 μ g of protein) isolated from control MLE-12 cells with appropriate controls, incubated for 30 min at 37°C, followed by were addition of streptavidin beads to immunodetect S1P bound to HDAC1 by Western blotting. Shown is a representative Western blot depicting binding of S1P to HDAC1. Human recombinant HDAC1 (10 ng/ml) was also used to detect S1P binding to the recombinant protein following the above procedure.

Figure 9. Phloretin, an aldehyde trapping agent, attenuates LPS- and *P. aeruginosa*-induced histone acetylation

HLMVECs (~95% confluence) grown in 35-mm dishes were pre-treated with phloretin (100 μ M) for 1 h prior to challenge with LPS (1 μ g/ml) or heat inactivated and lysed *P. aeruginosa* (PA) (1×10^8 CFU/ml) for 3 h, and cell lysates were subjected to Western blotting with anti-histone H3K9, anti-histone H4K8, anti-histone H3 and anti-histone H4 antibodies. Shown is a representative blot. Phloretin, the aldehyde trapping agent blocked LPS- or PA-induced H3 and H4 histone acetylation.

Figure 10. Role of 2-hexadecenal released from S1P by S1P lyase on HDACs and histone acetylation

S1P lyase is up-regulated in ALI by LPS, increasing the breakdown of S1P to 2-hexadecenal and ethanolamine phosphate. 2-hexadecenal, is a reactive long-chain fatty aldehyde, that can form adducts with proteins, lipids and DNA. Similar to 4-hydroxynonenal, 2-hexadecenal can interact with amino groups of lysine residues or interact with SH groups on HDACs to generate thionyl derivatives with HDACs. In the endothelial cells, modification of lysine or cysteine residue(s) by 2-hexadecenal can inhibit HDAC activity resulting in increased acetylation patterns of histones. Also, 2-hexadecenal, similar to 4-hydroxynonenal, can stimulate reactive oxygen species (ROS) production by activation of Nox proteins or mitochondria. The ROS can oxidize the SH groups on cysteines, and modulate the activity of HDACs. The mechanism of 2-hexadecenal mediated modulation of nuclear HDACs is unclear that needs further investigation.