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Am J Physiol Lung Cell Mol Physiol 301:L547-L556, 2011. First published 5 August 2011; doi:10.1152/ajplung.00058.2011

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Lysophosphatidic acid receptor 1 modulates lipopolysaccharide-induced inflammation in alveolar epithelial cells and murine lungs

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[1]*Department of Medicine, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania; Departments of* [2]*Pharmacology and* [3]*Medicine,* [4]*Institute for Personalized Respiratory Medicine, The University of Illinois at Chicago, Chicago, Illinois; and* [5]*Department of Molecular Biology, The Scripps Research Institutes, San Diego, California*

Submitted 25 February 2011; accepted in final form 29 July 2011

Zhao J, He D, Su Y, Berdyshev E, Chun J, Natarajan V, Zhao Y. Lysophosphatidic acid receptor 1 modulates lipopolysaccharide-induced inflammation in alveolar epithelial cells and murine lungs. *Am J Physiol Lung Cell Mol Physiol* 301: L547–L556, 2011. First published August 5, 2011; doi:10.1152/ajplung.00058.2011.—Lysophosphatidic acid (LPA), a bioactive phospholipid, plays an important role in lung inflammation by inducing the release of chemokines and lipid mediators. Our previous studies have shown that LPA induces the secretion of interleukin-8 and prostaglandin E_2 in lung epithelial cells. Here, we demonstrate that LPA receptors contribute to lipopolysaccharide (LPS)-induced inflammation. Pretreatment with LPA receptor antagonist Ki16425 or downregulation of LPA receptor 1 (LPA_1) by small-interfering RNA (siRNA) attenuated LPS-induced phosphorylation of p38 MAPK, I-κB kinase, and I-κB in MLE12 epithelial cells. In addition, the blocking of LPA_1 also suppressed LPS-induced IL-6 production. Furthermore, LPS treatment promoted interaction between LPA_1 and CD14, a LPS coreceptor, in a time- and dose-dependent manner. Disruption of lipid rafts attenuated the interaction between LPA_1 and CD14. Mice challenged with LPS increased plasma LPA levels and enhanced expression of LPA receptors in lung tissues. To further investigate the role of LPA receptors in LPS-induced inflammation, wild-type, or LPA_1-deficient mice, or wild-type mice pretreated with Ki16425 were intratracheally challenged with LPS for 24 h. Knock down or inhibition of LPA_1 decreased LPS-induced IL-6 release in bronchoalveolar lavage (BAL) fluids and infiltration of cells into alveolar space compared with wild-type mice. However, no significant differences in total protein concentration in BAL fluids were observed. These results showed that knock down or inhibition of LPA_1 offered significant protection against LPS-induced lung inflammation but not against pulmonary leak as observed in the murine model for lung injury.

lysophospholipid; signal transduction; lipopolysaccharide; inflammation; acute lung injury; lysophosphatidic acid receptor 1; interleukin-6

ACUTE LUNG INJURY (ALI) is characterized by acute respiratory failure resulting from destruction of the epithelium-capillary interface, inflammation, and extravasations of protein-rich fluid (14, 26, 35). The lung epithelium is the site of first contact for both inflammatory and inhaled physical environmental stimuli, acting as a physical barrier between lung interstitium and environment (30, 34). Disruption of epithelial barrier integrity results in paracellular leakage of relatively large proteins, such as albumin and immunoglobulin G, into alveolar spaces (15, 18, 19). Thus, the epithelium not only functions as a barrier but also secretes cytokines, chemokines, and lipid mediators to attract and activate a number of resident and infiltrating cells that play a role in airway inflammation (21, 25). Our previous studies have shown that bronchial epithelial cells release interleukin (IL)-8 (7, 43, 48), IL-6 (49), and prostaglandin E_2 (PGE_2) (17, 47) in response to bioactive lipid mediators and lipopolysaccharide (LPS), resulting in the infiltration of neutrophils into alveolar space (7, 47). The LPS-induced murine model of lung injury has been widely used to investigate mechanisms of ALI (18, 31, 40). Intratracheal administration of LPS increases protein and cytokine levels in bronchial alveolar lavage (BAL) fluids (18, 31, 40), whereas LPS challenge of lung endothelial (12, 23, 36) or bronchial epithelial (10, 18) cells enhances both barrier permeability and IL-6 release (3). The LPS-mediated cellular responses, such as activation of nuclear factor-κB (NF-κB) and phosphorylated (p)-38 MAPK, are through a receptor complex containing CD14 and Toll-like receptor 4 (TLR4) (16, 24, 33, 37). CD14 is known to be expressed in lipid rafts, which are plasma membrane microdomains that are enriched for cholesterol and sphingomyelin and characterized by insolubility in nonionic detergents (8, 28). In addition to interaction with LPS, recent studies show that CD14 binds to surfactant proteins (2, 4, 32) and integrin-β_1 (22), suggesting involvement of additional new signaling pathways in CD14-regulated LPS-induced inflammation.

Studies from our group and others show that lysophosphatidic acid (LPA) functions as both a pro- and anti-inflammatory mediator in inflammatory lung diseases (17, 43, 45–47). LPA induces IL-8 secretion in human bronchial epithelial cells and promotes infiltration of neutrophils into alveolar space (7, 43, 47, 48). Tager et al. (38) showed a critical role for LPA and LPA receptor 1 (LPA_1) in pathogenesis of pulmonary fibrosis (38). Furthermore, downregulation of LPA_2 reduced infiltration of eosinophils into airway lumen (47), suggesting that LPA and LPA receptors may exhibit proinflammatory properties. Also, recent evidence indicates that LPA plays an anti-inflammatory role by releasing IL-13 decoy receptor (IL-13Rα_2) (45) and PGE_2 (17) in lung epithelial cells. Intravenous (9) or intratracheal (18) administration of LPA reduces LPS-induced inflammation in the lung. The "Yin and Yang" effect of LPA in lung inflammation may be because of differences in preparations and targets of LPA; however, the roles of endogenous LPA and LPA receptors in LPS-induced acute lung inflammation are not clear.

The objective of this study was to determine the role of LPA receptors in LPS-induced acute lung inflammation. The data show for the first time that interaction between LPA_1 and CD14 regulates LPS-induced lung inflammation. Moreover,

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LPS increased LPA production and LPA_1 expression in vivo, and knock down of LPA_1 in mice ($LPA_1^{-/-}$) attenuated LPS-induced inflammation.

MATERIALS AND METHODS

Materials. 1-Oleoyl (18:1) LPA, Ki16425, LPS (*Escherichia coli* O127:B8), antibody to β-actin, and methyl-β-cyclodextrin (MBCD) were purchased from Sigma-Aldrich (St. Louis, MO). Antibodies to p-I-κB, p-I-κB kinase (IKK) α/β, IKKα/β, p38 MAPK, and p-p38 MAPK were from Cell Signaling Technology (Beverly, MA). Antibody to CD14 was from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody to LPA_1 was from Lifespan Bioscience (Seattle, WA). Horseradish peroxidase-conjugated goat anti-rabbit, anti-mouse, and real-time RT-PCR reagents were from Bio-Rad Laboratories (Hercules, CA). The enhanced chemiluminescence kit for detection of proteins by Western blotting was obtained from Thermo Fisher Scientific (Waltham, MA). All other reagents were of analytical grade.

Cell culture. The mouse lung epithelial cell line, MLE12, was purchased from American Type Cell Culture (Manassas, VA) and cultured in HITES medium with 10% FBS at 37°C in 5% CO_2. Three hours before experimentation, the normal medium was replaced with serum-free medium.

Small-interfering RNA tranfection. Control small-interfering RNA (siRNA) and LPA_{1-3} siRNA were purchased from Santa Cruz Biotechnology. siRNAs (50 nM) was transfected into MLE12 cells by electroporation (51). After 73 h, cell medium were replaced with serum-free medium, and cells were treated with LPS.

IL-6 measurement. BAL fluids or culture supernatants were centrifuged at 500 *g* for 10 min to remove cell debris. IL-6 levels were measured using an enzyme-linked immunosorbent assay (ELISA) kit for mouse IL-6 according to the manufacturer's instructions (Invitrogen).

Immunoblotting. Equivalent amounts of cell lysates (20 μg protein) were separated by 10% SDS-PAGE, transferred to polyvinylidene difluoride membranes, blocked with 5% (wt/vol) BSA in 25 mM Tris·HCl, pH 7.4, 137 mM NaCl, and 0.1% Tween 20 (TBST) for 1 h, and incubated with antibodies (1:1,000) in 5% (wt/vol) BSA in TBST for 1–2 h at room temperature (RT). The membranes were washed at least three times with TBST at 15-min intervals and then incubated with a horseradish peroxidase-conjugated rabbit or mouse secondary antibody (1:3,000) for 1 h at RT. The membrane was developed with the enhanced chemiluminescence detection system according to the manufacturer's instructions.

Transfection of adenoviral constructs. Infection of MLE12 cells (~60% confluence) with purified empty adenoviral vector and adenoviral vectors of mouse lipid phosphate phosphatase 1 (LPP-1) wild type were carried out in six-well plates as described previously (48). Following infection with different multiplicity of infection (MOI) in 1 ml of HITES medium for 48 h, the virus-containing medium was replaced with DMEM-F-12 medium, and experiments were performed.

Fluorescence immunostaining. MLE12 cells were grown in a glass chamber and treated with LPS for 2 h. Cells were then fixed with 3.7% of formaldehyde for 20 min, followed by permeabilization with 0.1% of Triton X-100 for 1 min. Localization of LPA_1 and CD14 was determined by immunofluorescence staining after incubation with primary antibodies and fluorescence-labeled secondary antibodies. Images were captured by a Nikon ECLIPSE TE 300 inverted microscope.

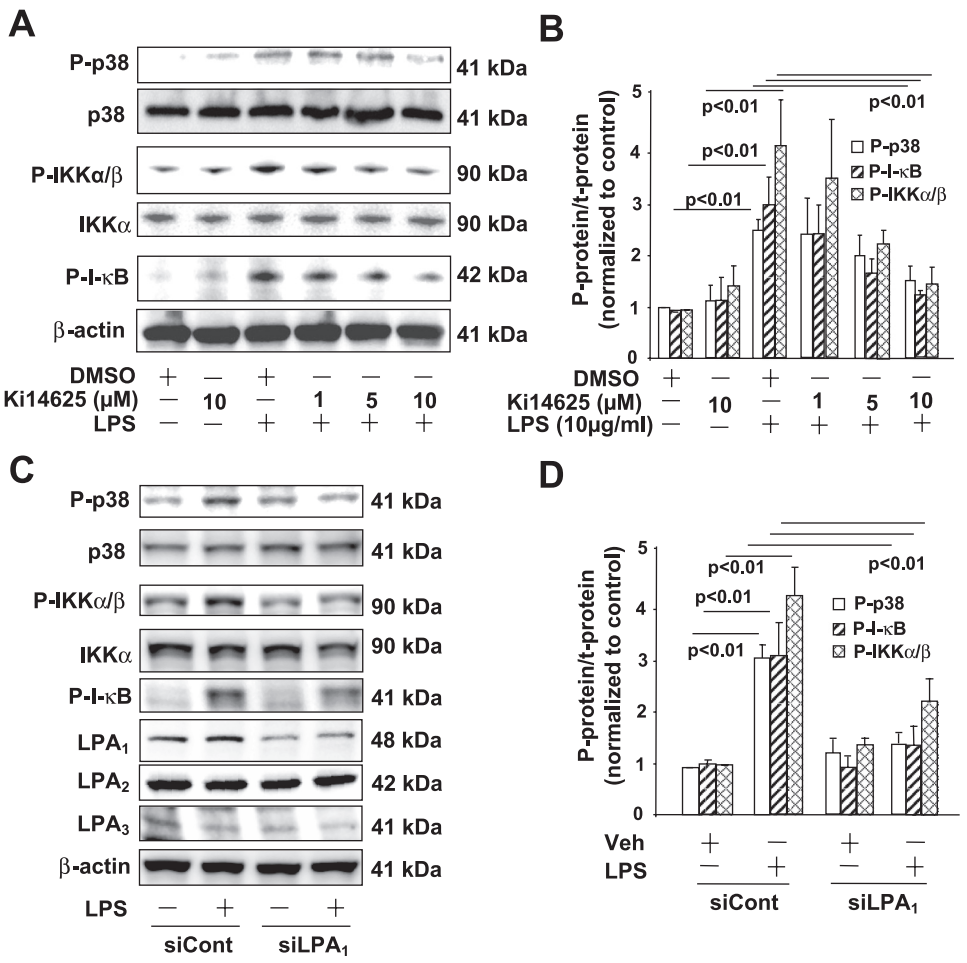

Fig. 1. Inhibition or downregulation of LPA_1 attenuates LPS-induced signaling. *A*: MLE12 cells were treated with Ki16425 (1, 5, and 10 μM, 1 h) before LPS challenge (10 μg/ml, 1 h). Equivalent amounts of cell lysates were subjected to immunoblotting with antibodies against phosphorylated (p)-p38, p-38, p-I-κB kinase (IKK), IKK, p-IκB, and β-actin. Representative blots from 3 independent experiments are shown. DMSO, dimethyl sulfoxide. *B*: relative expression levels of the above proteins as evidenced from density profiles using image J software. *C*: MLE12 cells were transfected with control small-interfering RNA [siRNA (siCont)] or LPA_1 siRNA ($siLPA_1$, 50 ng/ml, 72 h) before LPS challenge (10 μg/ml) for 1 h. Cell lysates were analyzed by immunoblotting using antibodies against p-p38, p-38, p-IKK, IKK, p-IκB, and β-actin. Representative blots from 3 independent experiments are shown. *D*: intensity changes of phosphoproteins in the immunoblots shown in *C* were analyzed by image J software. Veh, vehicle.

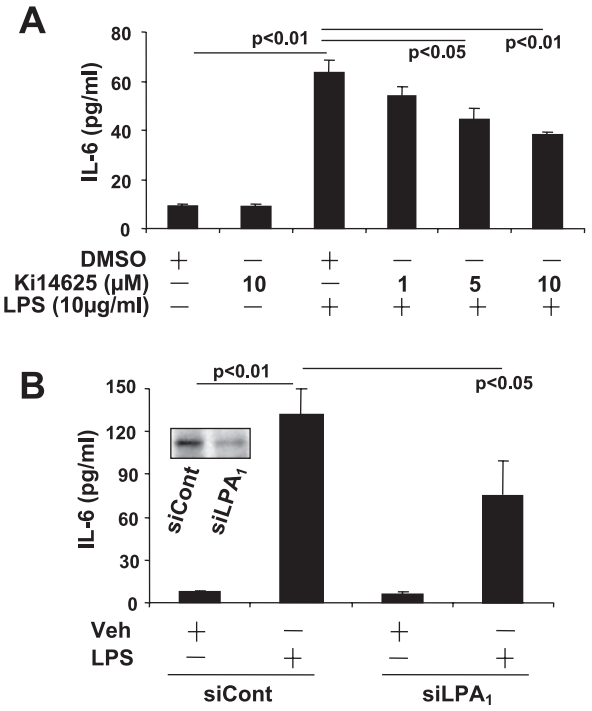

Fig. 2. Inhibition or downregulation of LPA_1 attenuates LPS-induced IL-6 secretion. *A*: MLE12 cells were pretreated with Ki16425 (1–10 μM, 1 h) before LPS challenge (10 μg/ml, 3 h). IL-6 levels in culture supernatants were measured by enzyme-linked immunosorbent assay (ELISA). *B*: MLE12 cells were transfected with siCont or $siLPA_1$ (50 ng/ml, 72 h) before LPS challenge (10 μg/ml) for 3 h, and IL-6 levels in culture supernatants were measured by ELISA. *Inset* shows the effect of $siLPA_1$ on expression of LPA_1 based on immunoblotting whole cell lysates using anti-LPA_1 antibody.

PCR-based genotyping of $LPA_1^{-/-}$ mice. The Extract-N-Amp Tissue PCR kit (Sigma-Aldrich) was used for isolating genomic DNA from mouse tail and amplifying LPA_1-specific DNA fragments. The primers for LPA_1 knockout mice were described in previous studies (5, 6).

LPS-induced ALI in a murine model. $LPA_1^{-/-}$ mice were generated as previously described (5, 6). All of the mice were bred and housed in a specific pathogen-free barrier facility maintained by the University of Chicago Animal Resources Center. Adult male, 8- to 10-wk-old mice with an average weight of 20–25 g were anesthetized with 3–5 ml/kg of anesthesia mixture of ketamine and of xylazine. LPS (5 mg/kg) in PBS or PBS alone was administered intratracheally. After 24 h, lungs were lavaged by an intratracheal injection of 1 ml of PBS solution followed by gentle aspiration; the lavage was repeated two times to recover a total volume of 1.8–2.0 ml. The recovered BAL fluids were processed for determining protein and IL-6 concentrations. Lungs from control and LPS-challenged mice were collected for histological evaluation by staining with hematoxylin and eosin (H&E). Plasma was collected, and LPA levels were measured by LC-MS/MS. All animal experiments were approved by the University of Chicago Institutional Animal Care & Use Committee (Chicago, IL) and adhered to strict humane treatment of experimental animals.

RNA extraction and real-time RT-PCR. Total RNA was extracted from lung tissue by TRIzol (Sigma) according to the manufacturer's instructions. RNA (1 μg) was reverse-transcripted using the cDNA synthesis kit (Bio-Rad), and real-time PCR was performed to assess expression of mouse LPA_{1-5} genes using specific primers as previously described (47). Amplicon expression in each sample was normalized to its 18S RNA content. The relative abundance of target mRNA in each sample was calculated based on the following formula: mRNA abundance $= 2^{-(\text{LPAR Threshold Cycle})}/2^{-(\text{18S Threshold Cycle})} \times 10^6$ where LPAR is LPA receptor.

LPA measurement by mass spectrometry. Lipids in plasma were extracted as described (13). In brief, LPA levels were determined using liquid chromatography and tandem mass spectrometry (LC) with an ABI-4000 Q-TRAP hybrid triple quadrupole/ion trap mass

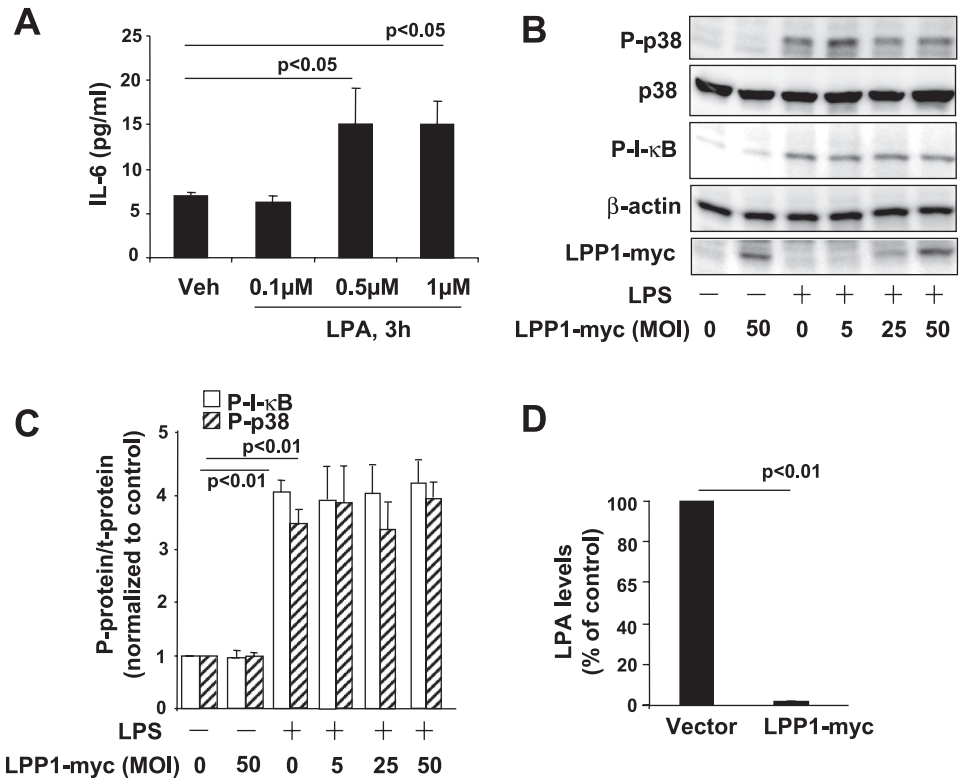

Fig. 3. LPS-induced phosphorylation of p38 MAPK and I-κB is independent of lysophosphatidic acid (LPA) generation. *A*: MLE12 cells were treated with LPA (0.1–1 μM) for 3 h, and IL-6 levels in culture supernatants were measured by ELISA. *B*: MLE12 cells (~60% confluence) were infected with adenoviral vector control or adenoviral mouse lipid phosphate phosphatase 1 (LPP-1) wild type at different multiplicities of infection (MOI) (0, 5, 25, or 50) for 48 h. The virus-containing medium was replaced with DEEM-F-12 medium, and cells were challenged with LPS (10 μg/ml) for 1 h. Equivalent amounts of cell lysates were subjected to Western blotting with antibodies against p-p38, p-38, p-IκB, β-actin, and Myc. Representative blots from 3 independent experiments are shown. *C*: relative expression levels of the above proteins as evidenced from density profiles using image J software. *D*: 1 μM of 18:1 LPA was added to cell cultures of MLE12 cells transfected with adenoviral vector control or adenoviral mLPP-1 wild type (50 MOI, 48 h) for 1 h. Supernatant was collected, and LPA levels in supernatant were measured by LC-MS/MS.

spectrometer (MS) coupled with an Agilent 1100 liquid chromatography system. Lipids were separated using methanol-water-HCOOH, 79:20:0.5, vol/vol/vol with 5 mM NH_4COOH as *solvent A* and methanol-acetonitrile-HCOOH, 59:40:0.5, vol/vol/vol with 5 mM NH_4COOH as *solvent B*. LPA molecular species were analyzed in negative ionization mode with declustering potential and collision energy optimized for LPA.

Statistical analyses. All results were subjected to statistical analysis using one-way ANOVA and, where appropriate, analyzed by Student-Newman-Keuls test. Data are expressed as means ± SD of triplicate samples drawn from a minimum of three independent experiments, and a value of $P < 0.05$ was considered statistically significant.

RESULTS

Involvement of LPA_1 in LPS-induced signaling and IL-6 secretion. MLE12 cells were pretreated with either dimethyl sulfoxide (DMSO, 0.1%) or a $LPA_{1\&3}$ antagonist, Ki16425 (1–10 μM, 1 h), and then challenged with LPS (10 μg/ml) for 1 h. Activation of p38 MAPK and NF-κB signaling was determined by immunoblotting using specific antibodies. As shown in Fig. 1, *A* and *B*, LPS stimulated phosphorylation of p38 MAPK, IKKα/β, and I-κB in MLE12 cells; however, pretreatment with Ki16425 attenuated LPS-mediated signal transduction of MAPK and NF-κB in a dose-dependent manner. LPS treatment had no effect on total p38 MAPK, IKKα, and β-actin expression. To confirm the role of LPA_1, we abrogated the expression of LPA_1 by transfecting cells with LPA_1 siRNA (50 nM, 72 h) before LPS challenge. Downregulation of LPA_1 expression attenuated LPS-induced phosphorylation of p38 MAPK, IKKα/β, and I-κB (Fig. 1, *C* and *D*). LPA_1 siRNA transfection reduced LPA_1 expression but had no effect on LPA_2 or LPA_3 expression (Fig. 1*C*). LPS induces proinflammatory cytokine release, including IL-6. We next investigated the effects of downregulation or inhibition of LPA_1 on LPS-induced IL-6 secretion in MLE12 cells. Consistent with the conclusion that LPS induces proinflammatory responses, LPS (10 μg/ml) significantly increased IL-6 secretion in MLE12 cells. Furthermore, pretreatment with Ki16425 (1–10 μM) for 1 h (Fig. 2*A*) or downregulation of LPA_1 by siRNA (Fig. 2*B*) reduced LPS-induced IL-6 secretion (25–43%). These results suggest a role of LPA_1 in LPS-mediated IL-6 secretion in mouse alveolar epithelial cells.

Overexpression of LPP1 has no effect on LPS-induced signaling. To investigate mechanisms of LPA_1-mediated LPS signaling, we first determined the role of LPA in IL-6 secretion

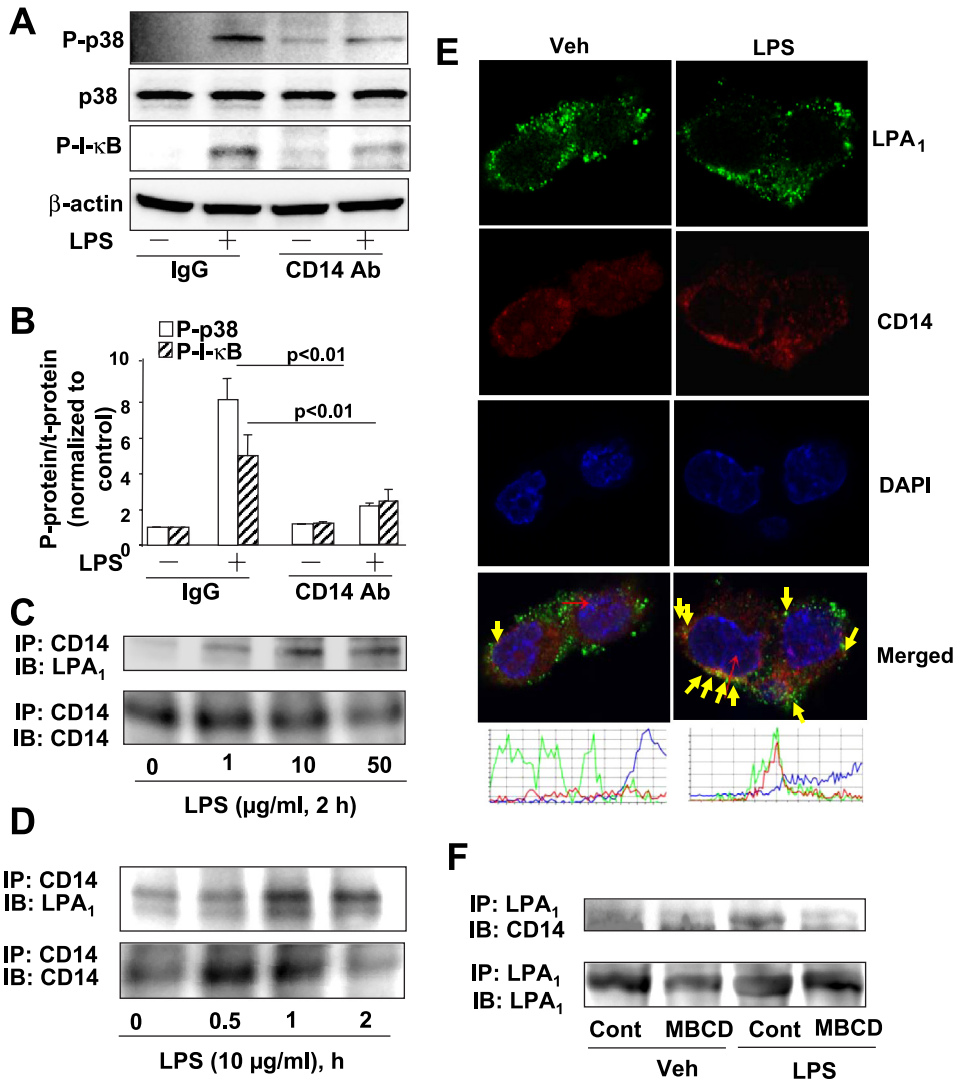

Fig. 4. LPS increases LPA_1 interaction with CD14. *A*: MLE12 cells were incubated with neutralizing CD14 antibody (Ab, 10 μg/ml) or an equivalent amount of IgG for 6 h before LPS (10 μg/ml, 1 h) challenge. Equivalent amounts of cell lysates were subjected to immunoblotting with antibodies against p-p38, p-38, p-IκB, and β-actin. Representative blots from 3 independent experiments are shown. *B*: relative expression levels of the above proteins as evidenced from density profiles using image J software. *C*: MLE12 cells treated with LPS (1, 10, or 50 μg/ml) for 2 h; CD14-containing protein complexes were immunoprecipitated (IP) using a CD14-specific antibody and analyzed by immunoblotting (IB) with antibodies to LPA_1 and CD14. Representative blots from 3 independent experiments are shown. *D*: MLE12 cells were treated with LPS (10 μg/ml) for 0.5, 1, and 2 h, CD14 immunoprecipitated with an anti-CD14 antibody, and analyzed for coimmunoprecipitated proteins. Representative blots from 3 independent experiments are shown. *E*: MLE12 were treated with 5 mM methyl-β-cyclodextrin (MBCD) for 2 h before LPS challenge (10 μg/ml) for 2 h. LPA_1 was immunoprecipitated with an anti-LPA_1 antibody and analyzed for coimmunoprecipitated proteins. Representative blots from 2 independent experiments are shown. *F*: MLE12 cells grown on a glass chamber were treated with LPS (10 μg/ml) for 2 h, fixed using 3.7% formaldehyde for 20 min, followed by a wash in PBS containing 0.1% Triton for 1 min. LPA_1 and CD14 were immunostained with antibodies to LPA_1 and CD14. Fluorescence intensity profiles are also shown. Green fluorescence is for LPA_1 signal, red fluorescence is for CD14 signal, and blue fluorescence is for nuclei signal. Arrows show colocalization of LPA_1 and CD14. Shown are representative data from 3 independent experiments.

in MLE12 cells. MLE12 cells were treated with LPA (0.1–1 μM) for 3 h, and IL-6 levels in culture supernatants were measured using an ELISA kit. As shown in Fig. 3*A*, LPA (0.5 and 1 μM) slightly induced IL-6 secretion (~2-fold) to a much lesser extent compared with LPA-induced IL-8 secretion (7, 17). To investigate whether ligation of LPA to LPA_1 is involved in LPS-induced signaling, overexpression of LPP1, a key enzyme to dephosphorylate LPA to monoacylglycerol (48), was performed before LPS treatment. Overexpression of *myc*-tagged LPP1 (5–50 MOI, 24 h) had no effect on LPS-induced phosphorylation of p38 MAPK and I-κB (Fig. 3, *B* and *C*); however, overexpression of LPP1 drastically reduced exogenously added LPA (98.01 ± 0.46% compared with control) (Fig. 3*D*). Furthermore, LPS treatment (10 μg/ml, 0–6 h) did not increase LPA generation in either medium or cells (data not shown). These data suggest that the attenuation effect of LPA_1 on LPS-mediated phosphorylation of p38 MAPK and I-kB is not mediated by LPA generation and that a LPA-independent mechanism may regulate the LPS signaling.

LPS induces LPA_1 interaction with CD14. CD14, as a coreceptor, regulates LPS-induced signal transduction (8, 28). To investigate the role of CD14, MLE12 cells were incubated with neutralizing CD14 antibody (10 μg/ml, 6 h), which attenuated LPS-induced phosphorylation of p38 MAPK and I-κB (Fig. 4, *A* and *B*). We have previously shown in lung epithelial cells that there is a cross talk between LPA receptor(s) and receptor tyrosine kinases such as epidermal growth factor receptor (EGF-R) (43) and c-Met (44). Therefore, we examined the potential interaction between LPA_1 and CD14. MLE12 cells treated with LPS revealed enhanced LPA_1 interaction with CD14 in a dose- and time-dependent fashion (Fig. 4, *C* and *D*), however, TLR4 was not detectable in LPA_1-immunoprecipitated complex (data not shown). Double-fluorescence immunostaining with antibodies to LPA_1 and CD14 revealed that LPS treatment (10 μg/ml, 2 h) increased colocalization of LPA_1 (green) and CD14 (red) on plasma membrane (yellow) (Fig. 4*E*). Both LPA_1 (39) and CD14 (8, 28) have been shown to be associated in lipid rafts, a functional domain on the cell surface. Disruption of lipid rafts by MBCD blocked the interaction between LPA_1 and CD14 (Fig. 4*F*). These results suggested that LPA_1 contributes to LPS-induced signaling through interaction with CD14 in lipid rafts.

LPS modulates expression of LPA receptors in MLE12 and mouse lungs. LPA receptors are widely expressed in various tissues. To investigate the effect of LPS on the expression of LPA receptors in lung tissue, C57/BL6 mice were instilled with LPS intratracheally (1 and 5 mg/kg) for 24 h, and RNA and protein were extracted from lung tissues. Compared with nonchallenged control mice, LPS treatment significantly increased mRNA levels of LPA_1 and LPA_2, without altering LPA_3 expression (Fig. 5, *A*–*C*); however, Western blotting showed that LPS challenge increased protein expression of $LPA_{1–3}$ in lung tissues (Fig. 5, *D* and *E*). The increase in LPA_3 protein expression without altering the mRNA expression may be the result of LPS-increased LPA_3 protein stability. In contrast to in vivo LPS challenge, LPS treatment (10 μg/ml, 3–20 h) of MLE12 cells had no effects on LPA receptor expression (data not shown). These results show that LPS modulates expression of LPA receptors in mouse lungs.

LPS challenge increases plasma LPA levels in mice. Our previous study has shown that intratracheal LPS challenge increased LPA levels in BAL fluids (42). Here, we investigated whether LPS challenge increases LPA levels in mouse plasma. After intratracheal LPS (5 mg/kg body wt) challenge for 24 h, LPA molecular species in plasma were detected and quantified by LC-MS/MS (39). As shown in Table 1, 16:0 LPA, 18:2

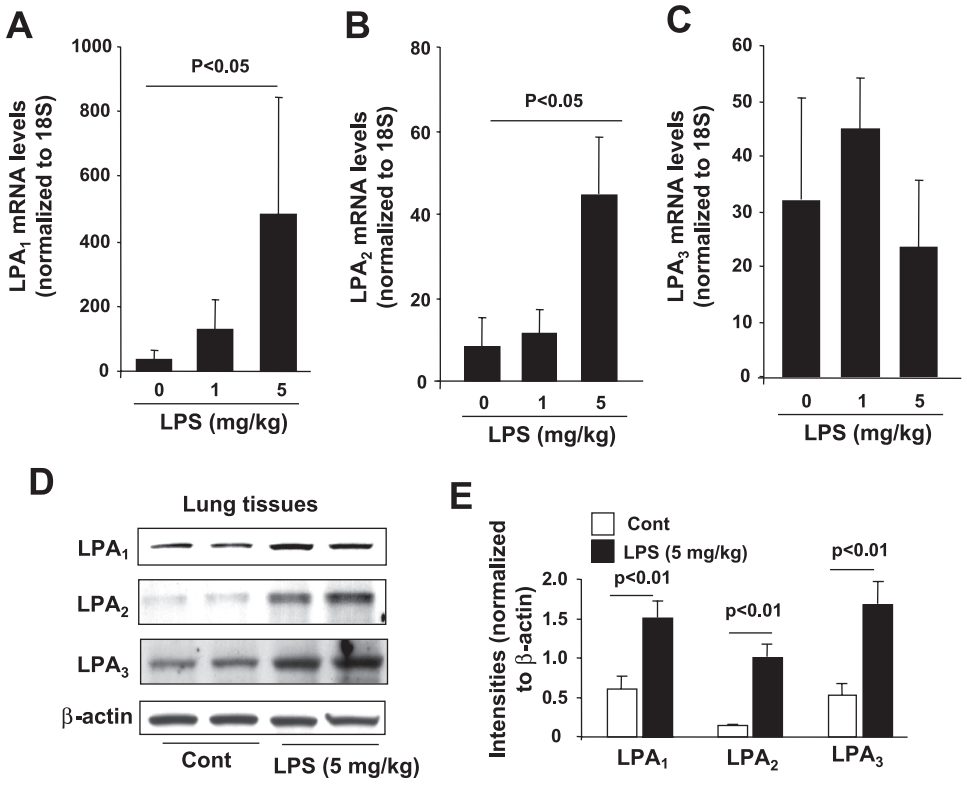

Fig. 5. LPS increases the expression LPA receptors in lung tissue. Mice were intratracheally challenged with LPS (5 mg/kg body wt) for 24 h. The lung tissue total RNA was extracted, and mRNA levels of LPA_1 (*A*), LPA_2 (*B*), and LPA_3 (*C*) were determined by real-time RT-PCR. $LPA_{1–3}$ protein expression were examined by immunoblotting (*D*). Relative expression levels of the above proteins as evidenced from density profiles using image J software.

Table 1. *Quantification of LPA molecular species in plasma*

LPA Species	PBS, pmol/ml	LPS Challenge, pmol/ml	*P* Value
14:0 LPA	5.99 ± 1.22	5.03 ± 0.75	0.048
16:1 LPA	0.99 ± 0.13	1.96 ± 0.23	0.004
16:0 LPA	16.54 ± 1.60	27.68 ± 1.52	0.001
18:2 LPA	40.59 ± 4.22	103.71 ± 9.53	0.000
18:1 LPA	7.32 ± 1.14	15.12 ± 1.48	0.002
18:0 LPA	11.81 ± 0.60	15.51 ± 0.46	0.001
20:5 LPA	1.40 ± 0.39	1.49 ± 0.25	0.841
20:4 LPA	30.49 ± 2.99	33.39 ± 2.81	0.451
20:3 LPA	3.33 ± 0.19	5.17 ± 0.81	0.056
20:2 LPA	0.50 ± 0.09	0.62 ± 0.03	0.231
22:6 LPA	42.08 ± 5.87	47.18 ± 3.23	0.425
22:5 LPA	3.90 ± 0.80	4.73 ± 0.39	0.338
22:4 LPA	0.58 ± 0.22	0.81 ± 0.11	0.338
22:3 LPA	0.12 ± 0.05	0.07 ± 0.02	0.391
22:2 LPA	0.12 ± 0.05	0.07 ± 0.03	0.351
Total LPA	165.70 ± 16.13	262.53 ± 19.22	0.003

Mice were intratracheally challenged with lipopolysaccharide (LPS, 5 mg/kg body wt) for 24 h. Plasma was collected, and lipids were extracted. Lysophosphatidic acid (LPA) molecular species were quantified by LC-MS/MS with 17:0 LPA as standard.

LPA, 20:4 LPA, and 22:6 LPA were abundant in mouse plasma, which is consistent with earlier findings (50). LPS challenge significantly increased plasma LPA levels to ~1.6-fold (control: 165.76 ± 16.13 pmol/ml; LPS: 262.53 ± 19.22 pmol/ml). Among the LPA molecular species, 18:2 LPA increased ~2.6-fold (control: 40.59 ± 4.22 pmol/ml; LPS: 103.71 ± 9.53 pmol/ml), 18:1 LPA increased ~2.1-fold (control: 7.32 ± 1.14 pmol/ml; LPS: 15.12 ± 1.48 pmol/ml), and 16:0 LPA increased ~1.7-fold (control: 16.54 ± 1.60 pmol/ml; LPS: 27.68 ± 1.52 pmol/ml); however, no significant changes were observed with respect to 22:6 LPA and 20:4 LPA. These results show that LPS challenge in mice modulates plasma LPA levels.

Knock down of LPA$_1$ in mice reduces LPS-induced acute inflammation. To investigate the role of LPA and LPA receptors in LPS-induced acute inflammation and injury, wild-type and LPA$_1$ knockout mice were challenged intratracheally with LPS (5 mg/kg body wt) for 24 h. BAL fluids were collected for IL-6, total cell number, and total protein levels. LPS challenge increased the secretion of IL-6 (Fig. 6*A*), total cell numbers in BAL fluids (Fig. 6*B*), and immune cell infiltration into alveolar spaces (Fig. 6*C*) in wild-type mice; however, knock down of LPA$_1$ attenuated LPS-induced IL-6 secretion (22–30%), total cell numbers in BAL fluids (~26%), and immune cell infiltration (Fig. 6, *A*–*D*). Elevated protein level in BAL fluids is an index of lung injury. As shown in Fig. 6*D*, LPS challenge increased BAL fluid total protein levels by ~6.0-fold in wild-type mice. Interestingly, the BAL fluid protein levels in LPS-challenged LPA$_1$ knockout mice were comparable to their wild-type counterparts (Fig. 6*D*). These results suggest that LPA and LPA$_1$ partly contribute to LPS-induced acute inflammation.

Ki16425 attenuates LPS-induced acute lung inflammation. To further substantiate the above observations, C57/BL6 mice were intratracheally injected with 25 μl of vehicle (0.1% DMSO PBS) or Ki16425 (5 μM dissolved in PBS containing 0.1% DMSO) 1 h before LPS challenge. Ki16425

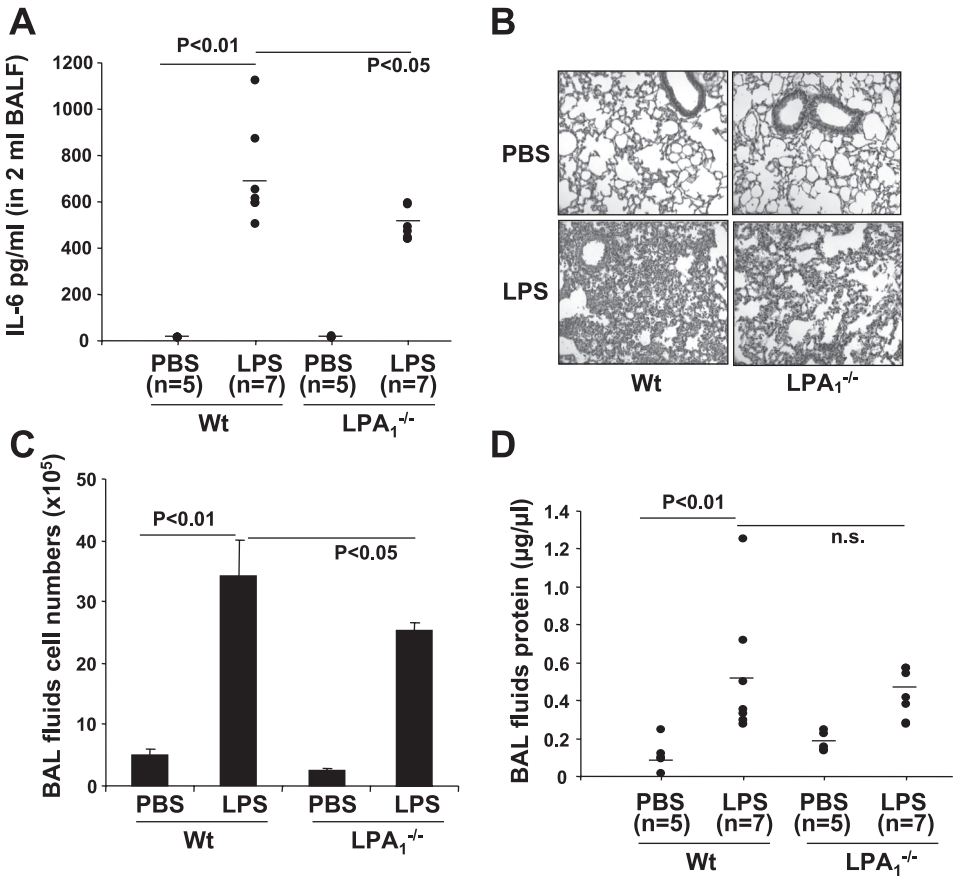

Fig. 6. LPA$_1^{-/-}$ mice are resistant to LPS-induced lung inflammation. Wild-type and LPA$_1^{-/-}$ mice were intratracheally challenged with LPS (5 mg/kg body wt) for 24 h. *A*: IL-6 levels in bronchoalveolar lavage fluids (BALF) were measured by ELISA. WT, wild type. *B*: lung tissues were stained with hematoxylin and eosin (H&E). Representative images are shown. *C*: cell counts in bronchoalveolar lavage (BAL) fluids were examined. *D*: the range of protein concentrations in BAL fluids and the means are shown. NS, not significant.

pretreatment reduced LPS-induced IL-6 secretion (~44%) (Fig. 7*A*), total cell numbers in BAL fluids (~24%) (Fig. 7*B*), and immune cell infiltration into alveolar spaces (Fig. 7*C*); however, Ki16425 had no effect on LPS-induced protein levels in BAL fluids (Fig. 7*D*). Notably, protein concentrations in BAL fluids in DMSO- or Ki16425-challenged mice were much higher compared with those from PBS-challenged mice. This may be because of the presence of DMSO in PBS, and Ki16425 was dissolved in DMSO. H&E staining does not support epithelial and endothelial barrier disruption by DMSO or Ki16425. These results suggest that inhibition of LPA_1 attenuates LPS-induced inflammation but not pulmonary leak.

DISCUSSION

The current study is aimed at determining the role of LPA_1 in endotoxin-induced inflammation using lung epithelial cells and a murine model of ALI. We found that, in lung epithelial cells, LPA_1 interacted with CD14, a coreceptor for LPS, in lipid rafts in response to LPS treatment. Inhibition or down-regulation of LPA_1 attenuated LPS-induced IL-6 secretion. These effects of LPA_1 may not involve the LPA-LPA_1 signaling axis, since reduction of extracellular LPA has no effect on LPS-induced phosphorylation of p38 MAPK and I-kB. LPS challenge increased LPA levels in plasma and BAL fluids and the expression of LPA receptors in lung tissues. Finally, in LPA_1 knockout mice or mice treated with LPA_1 antagonist, LPS-induced inflammation was partly reduced as evidenced by reduced IL-6 levels in BAL and infiltration of immune cells into the lung tissue.

In the lung, LPA levels are upregulated in BAL fluids of asthmatic patients (13), murine models of Th-2-mediated asthma (47), bleomycin-induced fibrosis (38), and LPS-induced ALI (42). Here, we show that plasma LPA levels are increased in a LPS-induced murine model of ALI. The mechanism(s) of LPA generation in BAL fluids is not clear; however, recent studies suggest a critical role of autotaxin in regulation of LPA levels in plasma and cancer tissues (1, 41), and our previous study shows that LPS challenge increased autotoxin levels in BAL fluids (42). LPA is known to signal through G protein-coupled LPA receptors. Although LPS has no effect on LPA receptor expression in MLE12 cells, intratracheal LPS challenge increased LPA receptor expression in lung tissues. It is possible that the LPS-induced increase in LPA receptor expression is the result of neutrophil influx. LPA receptors are expressed in neutrophils, and upregulation of LPA_1 and LPA_2 expression in neutrophils from pneumonia patients has been reported (29). Further studies are necessary to clarify the mechanisms of increased LPA receptor expression by LPS in lung tissues.

LPA exhibits both pro- and anti-inflammatory effects in airway diseases (46). The proinflammatory effects of LPA receptors in lung inflammatory diseases have been demonstrated (1, 38, 47). $LPA_1^{+/-}$- and $LPA_2^{+/-}$-deficient mice exhibited reduced inflammation in an ovalbumin-challenged murine model of asthma (47), and genetic knock down of LPA_1 (38) and LPA_2 (Zhao, unpublished data) protected mice from bleomycin-induced pulmonary fibrosis. Consistent with these findings, the current study showed that abrogation of LPA_1 either by using LPA_1 knockout mice or by inhibiting

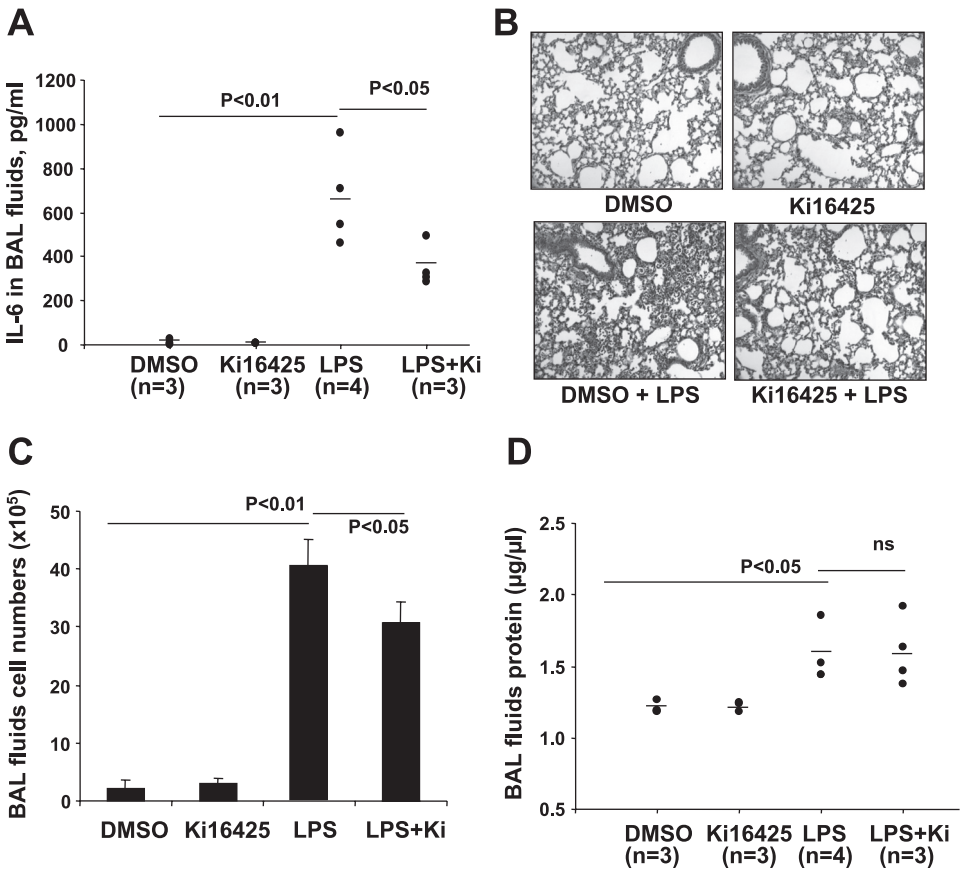

Fig. 7. Inhibition of LPA receptors reduces LPS-induced lung inflammation in vivo. C57/BL6 wild-type mice were intratracheally challenged with DMSO (0.1% in H_2O) or Ki16425 (Ki, 5 μM in 25 μl H_2O) before LPS challenge (5 mg/kg body wt it, 24 h). *A*: IL-6 levels in BAL fluids were measured by ELISA. *B*: lung tissues were stained with H&E. Representative images are shown. *C*: cell counts in BAL fluids were examined. *D*: the range of protein concentrations in BAL fluids are shown along with the means.

LPA_1 in wild-type mice attenuated LPS-induced inflammation. However, it did not prevent LPS-induced protein leakage in BAL fluids, thereby suggesting that vascular permeability is regulated by mechanisms that are independent of LPA_1. Although LPA has been shown to reduce permeability of human umbilical vein endothelial cells (20), LPA treatment of human pulmonary endothelial cells with lower expression of LPA receptors had no effects on permeability (Zhao, unpublished data). However, LPA treatment increased lung epithelial barrier integrity (18), suggesting LPA-mediated specific effects in the epithelium.

It is important to point out that exogenous LPA plays a protective role against endotoxin-induced lung injury. Intratracheal administration of LPA increased the innate immune response at early time points (<6 h) but not at later time periods (>12 h) (7), and LPA levels in BAL fluids rapidly returned to near-basal levels because of the short half-life of LPA (48). Furthermore, recent studies have shown that intravenous injection of LPA followed by intraperitoneal injection of LPS significantly increased mice survival, reduced the tumor necrosis factor-α level in BAL fluids, and decreased myeloperoxidase activity in lung by LPS (11). Intratracheal administration of LPA at 1 h after LPS challenge reduced LPS-induced inflammatory cell infiltration and IL-6 and protein levels in BAL fluids (18). These effects of exogenous LPA are in contrast to the effects of high levels of endogenous LPA, which are observed in BAL fluids or plasma from murine models of asthma (47) and ALI (42). These studies suggest that exogenous LPA plays a protective role by increasing transient innate immune responses in the early stage of inflammation, whereas a consistently high level of endogenous LPA promotes inflammation through induction of cytokine release and neutrophil influx via LPA receptor signaling and interaction with LPS/TLR4 signal transduction.

Both LPA and LPS stimulated secretion of cytokines, such as IL-6, in lung epithelial cells. We found that inhibition of LPA_1 attenuated LPS-induced activation of NF-κB and p38 MAPK, indicating a role of LPA_1 in LPS-mediated signaling. CD14, a coreceptor for LPS, is known to bind to LPS and LPS-binding protein (LBP) (37) and is wildly expressed in immune cells, including macrophages (37), neutrophils (37), and lung epithelial cells (37). We show here that CD14-neutralizing antibody attenuates LPS-induced signal transduction in MLE12 cells. Recent studies demonstrated that, in addition to LBP, CD14 interacted with other proteins, such as surfactant A (32), C (2), and D (32), integrin-β_1 (22), and Mac-1 (27); however, the biological role of these interactions has not been well investigated. Here, we demonstrated that inhibition of LPA_1 attenuated LPS-induced signaling pathways of phosphorylation of p38 MAPK and I-kB; however, reduction of LPA levels by overexpression of LPP1 had no effects on LPS-induced signals, indicating that LPA_1-mediated LPS signaling is independent on its LPA-LPA_1 signaling axis in cell culture conditions. However, our in vivo data show that LPS increases LPA generation in plasma and BAL fluids and LPA receptor expression in lung tissues, suggesting that LPA generation and the LPA-LPA_1 axis may regulate part of LPS signaling in the murine model of lung injury. This may because of involvement of other immune cells, such as neutrophils and macrophages, in lung tissues. Furthermore, we demonstrated that CD14 binds to LPA_1 in lipid rafts in response to LPS treatment, suggesting a novel role for LPA_1/CD14 in LPS-induced signaling via TLR4 in airway inflammation. LPA receptor(s) are known to interact with receptor tyrosine kinases, and we have shown that LPA receptors cross talk with EGF-R (52) and c-Met (53) in human bronchial epithelial cells, and LPA_1 also interacts with integrin-β_4 (42).

In summary, the current study for the first time demonstrated that LPA_1 is involved in LPS-induced activation of the NF-κB pathway, p38 MAPK, and IL-6 secretion through interaction with CD14, a coreceptor of LPS. In vivo experiments showed that LPS treatment increased LPA levels in plasma, and knock down of LPA_1 reduced LPS-induced IL-6 levels. These results support a significant role for LPA_1 in promoting LPS-induced acute lung inflammation.

ACKNOWLEDGMENTS

We thank Linnea Wallace and Daniel Mallampalli for excellent technical assistance.

GRANTS

This work was supported by National Heart, Lung, and Blood Institute grants HL-0911916 (to Y. Zhao), and HL-R37 079396 (to V. Natarajan) and the University of Pittsburgh Medical Center Start Up fund (to Y. Zhao).

DISCLOSURES

No conflicts of interest are declared by the authors.

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