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C1P Attenuates Lipopolysaccharide-Induced Acute Lung Injury by Preventing NF- κ B Activation in Neutrophils

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Recently, ceramide-1-phosphate (C1P) has been shown to modulate acute inflammatory events. Acute lung injury (Arnalich et al. 2000. *Infect. Immun.* 68: 1942–1945) is characterized by rapid alveolar injury, lung inflammation, induced cytokine production, neutrophil accumulation, and vascular leakage leading to lung edema. The aim of this study was to investigate the role of C1P during LPS-induced acute lung injury in mice. To evaluate the effect of C1P, we used a prophylactic and therapeutic LPS-induced ALI model in C57BL/6 male mice. Our studies revealed that intrapulmonary application of C1P before (prophylactic) or 24 h after (therapeutic) LPS instillation decreased neutrophil trafficking to the lung, proinflammatory cytokine levels in bronchoalveolar lavage, and alveolar capillary leakage. Mechanistically, C1P inhibited the LPS-triggered NF- κ B levels in lung tissue in vivo. In addition, ex vivo experiments revealed that C1P also attenuates LPS-induced NF- κ B phosphorylation and IL-8 production in human neutrophils. These results indicate C1P playing a role in dampening LPS-induced acute lung inflammation and suggest that C1P could be a valuable candidate for treatment of ALI. *The Journal of Immunology*, 2016, 196: 000–000.

Sphingolipids are structural molecules of eukaryotic cell membranes. However, they act as second messenger molecules in the regulation of cell homeostasis. Furthermore, sphingolipids were recently shown to contribute to the regulation of inflammatory responses and could modify the development and progression of human diseases (1, 2). A pathophysiological role of sphingolipids is implicated in diabetes, insulin resistance, neurodegenerative disorders, atherosclerosis, and allergic airway inflammation (1–5). The key molecule in the sphingolipid metabolism is ceramide, which regulates vital cellular functions such as apoptosis, cell growth, and differentiation. An important metabolite is ceramide-1-phosphate (C1P), which is generated through direct phosphorylation of ceramide by ceramide kinase (CerK) (2, 5, 6). Although CerK is the only enzyme described for the generation of C1P so far, there is convincing evidence for the existence of a CerK-independent metabolic pathway (7). There are still detectable levels of C1P in CerK-deficient animals (8) and in baculovirus-infected Sf9 cells treated with

CerK inhibitor (9). Notably, although C1P was identified >20 y ago, some of its biological functions have only been revealed in the last few years. C1P has been shown to act either as an intracellular second messenger (10) or as an extracellular mediator binding to a recently functionally identified, but still not cloned, specific G protein-coupled receptor upon secretion to the extracellular milieu. This specific C1P-coupled receptor was discovered and described only on RAW 264.7 macrophages yet (7, 11). It has been shown that C1P regulates cell proliferation and apoptosis (1–3, 5–7, 10–12). In addition, there is increasing evidence that C1P plays a role in inflammatory responses, because it stimulates phagocytosis of neutrophils (8, 13), induces migration of macrophages (11, 14), as well as synthesis of eicosanoids (15), and modulates cytokine production by macrophages and mast cells (1, 5, 16, 17). Initial studies demonstrated an increase of ceramide concentration in apoptotic cells after stimulation of J774 macrophages with LPS (16). Moreover, an anti-inflammatory effect of C1P was shown in LPS-stimulated HEK 293 cells and human PBMCs (18).

Acute lung injury (ALI) (19) occurs in the setting of an acute severe illness accompanied by systemic inflammation (20, 21) and is characterized by diffuse parenchymal pulmonary inflammation and edema (20–23). Intratracheal (i.t.) and i.p. instillation of LPS, a bacterial cell wall component, to rodents is a well-accepted and common experimental model for ALI from pulmonary and extrapulmonary origin (24, 25) featuring key pathological components of the disease such as profound neutrophilic lung recruitment, vascular leakage/lung edema, and subsequent systemic inflammation (20, 26). Unfortunately, despite marked efforts and multiple therapeutic strategies, the mortality rate of patients suffering from ALI remains high (21, 27–29).

In this study, we used an LPS-induced mouse model of ALI to investigate the effect of C8-C1P and C16-C1P treatment in a prophylactic and therapeutic setting. To adapt our observations to a human situation, we treated LPS-stimulated human blood isolated neutrophils with C1P in vitro. As a potential pathomechanistic link, the modulation of the NF- κ B pathway by C1P was studied.

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Abbreviations used in this article: ALI, acute lung injury; BALF, bronchoalveolar lavage fluid; CerK, ceramide kinase; C1P, ceramide-1-phosphate; i.t., intratracheal; KC, keratinocyte-derived chemokine; *Mmp-9*, matrix metalloproteinase 9; qPCR, quantitative PCR; Traf2, TNFR-associated factor 2.

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Materials and Methods

Reagents

Two types of C1P differing in carbonyl-chain length were used in our experiments. Natural C16-C1P was purchased from Matreya LLC (Pleasant Gap, PA) and solubilized in sterile nanopure water on ice using a probe sonicator as previously described (14). Synthetic C8-C1P was acquired from Cayman Chemical (Ann Arbor, MI) in a 1% solution of C8-C1P in ethanol. The solution was aliquoted and frozen at -20°C . Cells and mice were not affected through the maximum concentration of 0.0005% ethanol 99%. Vehicle control means equally diluted in ethanol concentration in PBS or medium in comparison with the diluted ethanol concentration of C8-C1P.

Animal studies

All experiments were approved by the local animal ethics committee and conducted according to the Helsinki convention for the use and care of animals. Male C57BL/6 mice (6–8 wk old) were bred at the animal facility of the University Hospital Freiburg under specific pathogen-free conditions. All animal experiments were performed using five animals per group and were repeated three times.

LPS model of acute lung inflammation. First, mice were anesthetized using ketamine (10 mg/kg; Intervet; Bela-Pharm GmbH, Vechta, Germany)/rompun (150 mg/kg; Bayer, Leverkusen, Germany) solution injected i.p. Second, they received an i.t. injection of LPS (300 $\mu\text{g}/\text{kg}$; *Escherichia coli* Serotype O26:B6; Sigma-Aldrich GmbH, Steinheim, Germany) diluted in 50 μl sterile PBS. In addition, indicated concentration of C1P or vehicle diluted in 80 μl sterile PBS was administered i.t. 1 h before (prophylactic study) or 24 h after (therapeutic study) the LPS application. Mice were euthanized 24 h after the last i.t. application.

Bronchoalveolar lavage fluid analysis by flow cytometry. Lungs were washed three times through i.t. cannulae with 1 ml PBS containing 0.5 mM EDTA. Total cell number was evaluated and differential cell distribution was determined by flow cytometry (FACSCalibur; BD Biosciences, San Diego, CA), by using the software CellQuest version 3.3 (BD Biosciences) and FlowJo version 10 (Tree Star, Ashland, OR) as previously described (30). In brief, mouse bronchoalveolar lavage fluid (BALF) cells were incubated with unlabeled anti-CD116/CD32 to block Fc receptors and stained for 20 min with anti-CD11c allophycocyanin, anti-Ly-6G (Gr-1) FITC, anti-CD3e PE-Cy7, anti-CD45R (B220) PE-Cy7, and anti-mouse F4/80 PE (all from eBioscience, Frankfurt, Germany), in PBS containing 0.5% BSA and 0.1% sodium azide. Gating strategy was as follows: first, non-erythrocytes have been gated using forward scatter and side scatter. Next, a lymphocyte gate was defined based on FCS and $\text{CD3}^+\text{B220}^+$ PE-Cy7 fluorescence. Neutrophils were determined as Gr-1^+ , $\text{CD3}^-\text{B220}^-$ cells, whereas macrophages were defined as F4/80^+ , CD11c^+ , and $\text{CD3}^-\text{B220}^-$ population. The percentages obtained for each cell type were multiplied by the number of total BALF.

Cytokine analysis. The levels of IL-1 β , IL-6, IL-8, MIP-2, keratinocyte-derived chemokine (KC), IFN- γ , and TNF- α were measured in BALF and neutrophil cell culture supernatants using ELISAs (Duoset; R&D Systems, Minneapolis, MN), according to the manufacturer's instructions. The detection limit was 2 pg/ml. Samples with values below the detection limit were assigned 1 pg/ml as cytokine concentration.

Plasma leakage assay. Plasma vascular leakage was examined as previously described (31). In brief, Evans blue dye conjugated to albumin (20 mg/kg) was injected into the tail vein of mice. Thirty minutes later, the mice were sacrificed and the lungs were perfused with PBS supplemented with 5 mM EDTA. Perfused lungs were excised en bloc, dried, weighed, and snap frozen in liquid nitrogen. The whole lung was homogenized in PBS (1 ml/100 μg tissue) before incubation in formamide at 60°C for 18 h. The OD of the supernatant was determined spectrophotometrically at 620 nm after centrifugation at $5000 \times g$ for 30 min. The concentration of the extravasated Evans blue in lung homogenate was calculated against the standard curve, and the results were expressed as microgram of Evans blue dye per gram lung tissue (31).

Histology. Frozen lung tissue sections were cut and stained with H&E. The density of neutrophils in the lung parenchyma was obtained as previously described (32–35). In brief, 20 photomicrographs of lung parenchyma (excluding areas of pulmonary vessels) were randomly obtained at $\times 400$ magnification. The area of the whole photograph and the area of light (air area) were calculated using the Software Image Pro Plus 4.0 (Media Cybernetics, Rockville, MD). By subtracting the air area from the total photograph area, we obtained the parenchyma tissue area. Then the number of neutrophils was counted in the tissue area according to the morphological criteria. The results were depicted as number of neutrophils per square millimeter of parenchymal tissue (neutrophils/ mm^2).

Immunofluorescence and neutrophil quantification. Four-micrometer-thick frozen lung sections were placed on polysine slides (Thermo Scientific), air-dried, and fixed with cold acetone for 10 min. Tissue sections were washed with PBS and blocked 1 h at room temperature with 5% goat serum, 1% BSA, 0.1% cold fish skin gelatin (Sigma-Aldrich), 0.1% Triton X-100, and 0.05% Tween 20 in TBS. Sections were incubated with 1:1000 diluted GR-1 Ab (clone RB6-8C5; BioLegend) for 1 h at room temperature. As secondary Ab, goat anti-rat Alexa555 (Life Technologies) was used. DAPI was added during the last 10 min of secondary Ab incubation. Fluoromount mounting medium (Sigma) and coverslips (36) were used to finish the preparation. For imaging, Axioplan2 microscope with $63\times$ oil immersion objective, AxioCam, and HAL100 have been used (all from Zeiss). For image acquisition and analysis, Axiovision software v4.9.1.0 (Zeiss) was used. Twelve high-power vision fields per lung were used for counting neutrophils.

Total and phosphorylated NF- κB expression in lung tissue. Lungs were homogenized in radioimmunoprecipitation assay buffer, containing PMSE, sodium orthovanadate, phosphatase inhibitor mixture B, and protease inhibitor mixture (Santa Cruz Biotechnology, Santa Cruz, CA) on ice for 15 min and centrifuged at $17,000 \times g$ at 4°C for 15 min to remove the cell debris. The amount of proteins was quantified by Quick Start Bradford Protein Assay (Bio-Rad Lab GmbH, Munich, Germany). For all samples, 50 μg protein was loaded in NuPAGE 4–12% Bis-Trigel (Invitrogen AG, Carlsbad, CA) and transferred to nitrocellulose membrane. The membrane was blocked with 5% milk powder and incubated with a primary Ab against phospho-NF- κB p65 (1:500, rabbit monoclonal IgG; Cell Signaling, Danvers, MA) overnight. Afterward we used HRP-conjugated secondary Abs against rabbit IgG (Cell Signaling) for 1 h. Primary Ab was visualized and enhanced by chemiluminescence with SuperSignal West Dura (Thermo Scientific, Rockford, IL). The amount of mouse β -actin detected by monoclonal anti-actin Ab clone C4 served as loading control (MP Biomedicals LLC, Solon, OH). The densitometric analysis was performed using ImageJ.

RNA isolation, cDNA synthesis, and quantitative PCR. Total RNA was isolated with QIAzol lysis reagent for gene expression analysis (QIAGEN GmbH, Hilden, Germany) following the manufacturer's protocol. cDNA synthesis was carried out using the First Strand cDNA synthesis kit (Thermo Fisher Scientific GmbH, Schwerte, Germany). Quantitative PCR (qPCR) was performed on a LightCycler 480 (Roche Diagnostic GmbH, Mannheim, Germany) using qPCR SYBR Green mix (Thermo Fisher Scientific GmbH). β_2 -MICROGLOBULIN and GAPDH served as reference genes. For all reactions the annealing temperature was 60°C . Primer design and relative quantifications were done as previously described (37); primer sequences are available upon request.

In vitro studies

Human neutrophil isolation and culture. Human blood neutrophils were obtained from venous blood using human Pancoll gradient (PAN-Biotech GmbH, Aidenbach, Germany) as previously described (38). Isolated neutrophils were resuspended in PBS and their purity determined by using Giemsa staining ($>98\%$). Neutrophils were seeded in 24-well plates ($2 \times 10^6/\text{well}$) in RPMI 1640 supplemented with 10% FCS and penicillin/streptomycin, and incubated at 37°C with 5% CO_2 in a humidified atmosphere. Neutrophils were stimulated with C1P (1, 10 μM) or vehicle for 1 h before and 1 h after LPS (1.5 $\mu\text{g}/\text{ml}$) stimulation. Finally, supernatant was collected 6 h after the last treatment and analyzed for IL-8 concentration.

NF- κB phosphorylation assay. Human neutrophils were seeded in six-well plates ($5 \times 10^6/\text{well}$) in RPMI 1640 with 0.1% FCS and penicillin/streptomycin. One hour before a 15-min LPS stimulation (1.5 $\mu\text{g}/\text{ml}$), cells were preincubated with C1P 1 μM . Subsequently, collected cells were washed in cold PBS and proteins were extracted using radioimmunoprecipitation assay lysis buffer as described earlier. Equal amounts of proteins (20 μg) were analyzed for β -Actin and phospho-NF- κB p65 (Ser⁵³⁶) (rabbit monoclonal IgG; Cell Signaling).

Statistical analysis. If not stated otherwise, groups were compared using one-way ANOVA, followed by Bonferroni comparison test (GraphPad Prism 5 Software, San Diego, CA). The p values <0.05 were regarded as significant.

Results

Effects of C1P on LPS-induced ALI

i.t. instillation of LPS (300 $\mu\text{g}/\text{kg}$) in C57BL/6 for 24 or 48 h resulted in severe acute lung inflammation, as demonstrated by increased BALF cell numbers (neutrophils and macrophages),

elevated BALF-cytokine levels (Figs. 1, 2), plasma leakage into the lungs (Fig. 3), and leukocyte infiltration in lung parenchyma (Fig. 4). The prophylactic administration of both 1 and 10 μ M C1P, 1 h before LPS administration, resulted in lower numbers of neutrophils and macrophages in BALF (Fig. 1A), decreased levels of proinflammatory cytokines, notably KC and MIP-2 (Fig. 1B), IL-6, and TNF- α (Fig. 1C), and significant reduction in microvascular plasma leakage to the lung (Fig. 3A). This was accompanied by a decreased amount of neutrophils (Fig. 4A, 4E) and inflammatory infiltrate in lung parenchyma (Fig. 4C).

Interestingly, there seems to be a dose response for C8-C1P, but not for C16-C1P. Especially in the prophylactic LPS model, cytokine levels of MIP-2 or IL-6 are more reduced by the treatment of C8-C1P 10 μ M instead of C8-C1P 1 μ M. Administration of C16-C1P independent of the concentration showed similar effects.

Notably, the beneficial effects of C1P on the cardinal features of LPS-induced lung inflammation were also observed when animals were treated with C1P 24 h after the administration of LPS-induced ALI (Figs. 2, 3B). Histological examination of the lungs with H&E and immunohistochemistry staining with Gr-1 for neutrophils from mice treated with LPS for 48 h showed a significantly higher number of neutrophils in the lung parenchyma than in the lungs of C1P-treated mice (Fig. 4B, 4D, 4F), suggesting that C1P can not only prevent but also treat established ALI. In addition, previous experiments have shown that C1P reduced the early inflammatory response after an LPS stimulation of 6 h (data not shown).

C1P reduced phosphorylation of NF- κ B p65 expression in lung tissue

LPS-induced lung injury has been reported to increase NF- κ B activation in the lungs (39). Thus, we next questioned whether C1P can decrease LPS-induced NF- κ B activation in the prophylactic and therapeutic settings of ALI. As shown in Fig. 5A, C16-C1P and C8-C1P 1 μ M significantly reduced LPS-induced NF- κ B p65 activation in the prophylactic model. Similar effects of C1P are shown on LPS-induced phospho-NF- κ B p65 in the therapeutic setting.

C1P attenuated mRNA NF- κ B2 expression in lung tissue

Furthermore, qPCR analysis revealed that the protective role of C1P can also be linked to the reduced mRNA expression of NF- κ B2

(Fig. 6A, 6D) and NF- κ B target genes including *CD83 molecule (CD83)* and *matrix metalloproteinase 9 (Mmp-9)* (Fig. 6A, 6D), *CCR5* and *IL-6* (Fig. 6B, 6E), *Myd88* (Fig. 6B), *TNFR-associated factor 2 (Traf2)* (Fig. 6E), and *Foxp3* (Fig. 6C, 6F). Myd88 plays a key role in the innate and adaptive immune response and is included in the activation of several proinflammatory genes. Especially mRNA level of *IL-6* was highly induced by LPS and attenuated with prophylactic and/or therapeutic C1P treatment. Reduced *IL-15* mRNA by C1P leads to lower *STAT3* mRNA, which is involved in many cellular processes such as cell growth and apoptosis (Fig. 6C, 6F). To conclude, C1P reduced the expression of LPS-induced genes, which are involved in the regulation of NF- κ B signaling.

Effects of C1P on LPS-induced NF- κ B activation and IL-8 production in neutrophils

Neutrophil recruitment and activation play a pivotal role in the pathophysiology of ALI; thus, we investigated the effects of C1P on LPS-induced IL-8 secretion and NF- κ B activation in purified human blood neutrophils.

According to our in vivo study, we determined the prophylactic and the therapeutic effect of C1P on the LPS-induced IL-8 production. Thus, neutrophils were treated with C1P (1 or 10 μ M) either 1 h before or 1 h after LPS stimulation (1.5 μ g/ml). Supernatants were collected after an additional 7 h. As shown in Fig. 7, C1P administration before (Fig. 7A) or after (Fig. 7B) LPS stimulation significantly decreased LPS-induced IL-8 production.

For the latter, neutrophils (5×10^6) were incubated with C1P or vehicle 60 min before stimulation with LPS for an additional 15 min. Pretreatment of neutrophils with C1P before LPS pulsing led to significant reduction in phospho-NF- κ B p65, compared with vehicle-treated, LPS-stimulated neutrophils, so that treatment with C1P significantly reduced the NF- κ B activation (Fig. 7C). In Fig. 7D we present a representative Western blotting for phospho-NF- κ B and β -actin.

Discussion

Sphingolipids have been shown previously to act as proinflammatory or anti-inflammatory agents (18, 40). Although the role of sphingosine-1-phosphate in LPS-induced lung inflammation has been extensively studied (12, 31, 41), the influence of

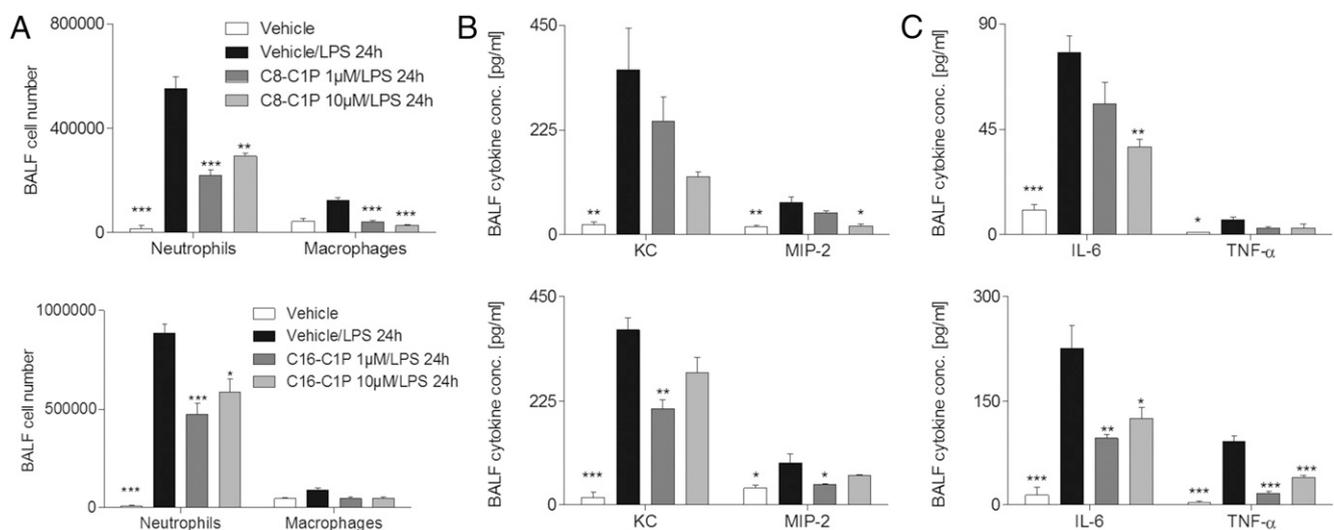


FIGURE 1. Prophylactic administration of C1P (1 and 10 μ M) before LPS administration significantly reduced ALI. C8-C1P (upper panels) and C16-C1P (lower panels) (1 and 10 μ M) were given 1 h before LPS administration and the animals were euthanized 24 h after LPS instillation. BALF cell differential count was measured by flow cytometry (A). Concentration of KC, MIP-2 (B) and IL-6, TNF- α in BALF (C) were determined by ELISA. One representative experiment out of three is shown. Values are given as mean \pm SEM. $n = 5$ mice in each group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus vehicle/LPS.

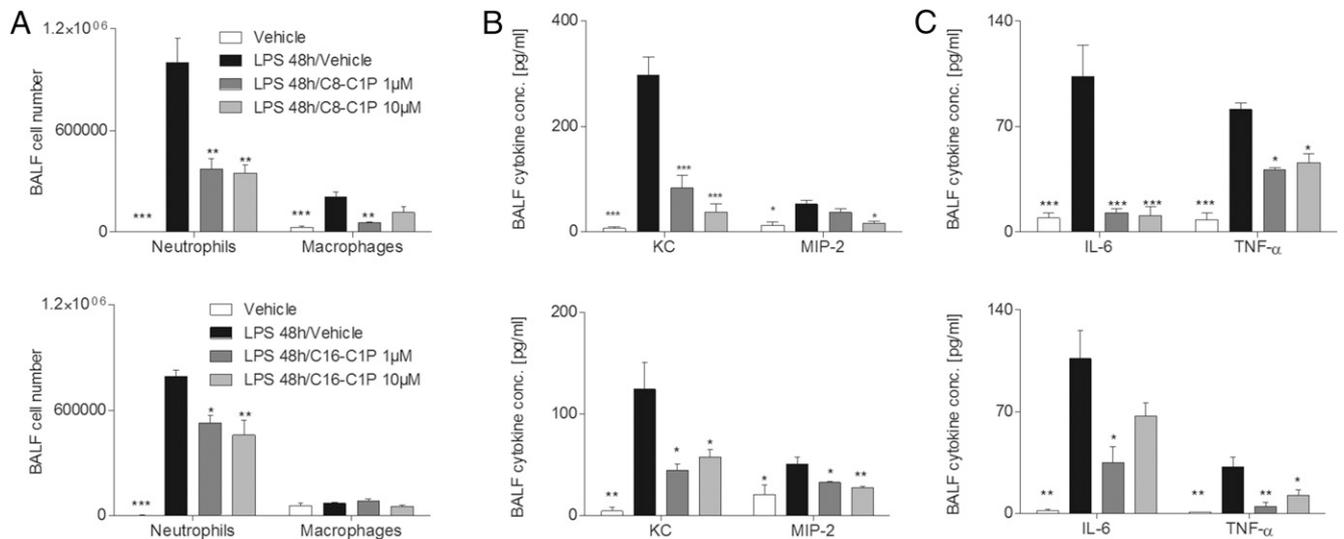


FIGURE 2. Therapeutic administration of C1P (1 and 10 μM) after LPS administration significantly reduced ALI. C8-C1P (upper panels) and C16-C1P (lower panels) (1 and 10 μM) were given 24 h after LPS administration. Animals were euthanized 48 h after LPS instillation. BALF cell differential count was analyzed by flow cytometry (A). KC, MIP-2 (B), IL-6, and TNF- α were determined in BALF by ELISA (C). One representative experiment of three is shown. Values are given as mean \pm SEM. $n = 5$ mice in each group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus vehicle/LPS 48 h.

C1P in this process is not well-known. Moreover, the exact pathogenesis and molecular mechanisms leading to ALI (19) are still poorly understood (20, 21, 42, 43). Hence specific therapies have not been identified yet, and the current management for ALI is mainly supportive (20, 21, 43). Our present study concentrates on C1P as an anti-inflammatory modulator of LPS-induced acute lung inflammation. A low-dose LPS model was used to reproduce important biomarkers of ALI, such as edema formation and proinflammatory cytokines release, according to the recommendations from the American Thoracic Society (44).

In this article, we demonstrate for the first time, to our knowledge, that both natural C16-C1P and the synthetic C8-C1P analog attenuate LPS-induced ALI in mice. Intrapulmonary application of C1P, before or after LPS administration, reduces the amount of neutrophils and the production of proinflammatory cytokines, and enhances the vascular leakage in the lung. Mechanistically we provide evidence that C1P inhibits LPS-induced *NF- κ B* mRNA expression and *NF- κ B* activation in lung tissue in vivo and neutrophils in vitro, and reduces LPS-primed IL-8 production by neutrophils.

ALI is characterized by pulmonary inflammation resulting from microvascular endothelial barrier failure followed by a rich protein pulmonary edema accumulation (20, 21, 26, 45, 46). Especially the number of neutrophils seems to play a key role in the severity of ALI (47). The intrapulmonary administration of LPS in rodents has been accepted as a clinically relevant model of ALI (20, 26). i.t. exposure of mice to LPS results in a massive recruitment of

neutrophils to the lungs as seen by increased BALF-neutrophilia at 24 and 48 h after LPS administration. The intrapulmonary treatment of mice with C1P significantly reduces BALF-neutrophilia. Furthermore, histological examination of the lungs from mice treated with LPS and immunohistochemistry staining with Gr-1 display a higher amount of neutrophils in the lung parenchyma in comparison with mice that received C1P in a prophylactic or therapeutic setting. In addition, LPS-induced proinflammatory cytokines, such as KC, MIP-2, IL-6, and TNF- α , which actively participate in the pathogenesis of ALI (e.g., by contributing to inflammatory cell recruitment, activation, and migration) (20, 26, 48, 49), were attenuated by C1P. Our findings were supported by Józefowski et al. (16), who described C1P as a negative regulator of TNF- α production by LPS in macrophages. Finally, IL-8 and its two homologs, KC and MIP-2 in mice, are important for the recruitment and activation of neutrophils (20, 26, 50) in ALI. IL-8 levels in BALF correlate with the severity and prognosis of the disease (20, 50). Thus, our observation that C1P inhibits these major cytokines involved in the induction and maintenance of LPS-induced ALI underlines the potency of this compound.

Nevertheless, differences are visible between C8-C1P concentrations, although C16-C1P independent of the concentration is more stable in the effect. One explanation could be the natural origin of the C16-C1P compound. Further pharmacokinetic and pharmacodynamics studies could help to fully understand how those active compounds were adsorbed and distributed in the body to their capability to influence the immune system.

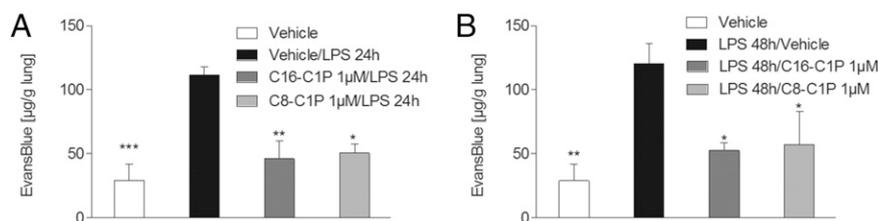


FIGURE 3. Prophylactic and therapeutic C1P treatment reduced LPS-induced plasma leakage. C1P (1 μM) was administered 1 h before LPS or 24 h after LPS instillation. In the prophylactic study (A), animals were euthanized 24 h and in the therapeutic study (B) 48 h after LPS administration. Plasma leakage was determined spectrophotometrically 18 h after Evans blue dye albumin (20 mg/kg) was injected into the tail vein. Values are given as mean \pm SEM. $n = 5$ mice in each group. The experiment was performed twice. * $p < 0.05$, ** $p < 0.01$ versus vehicle/LPS 24 h or versus LPS 48 h/vehicle.

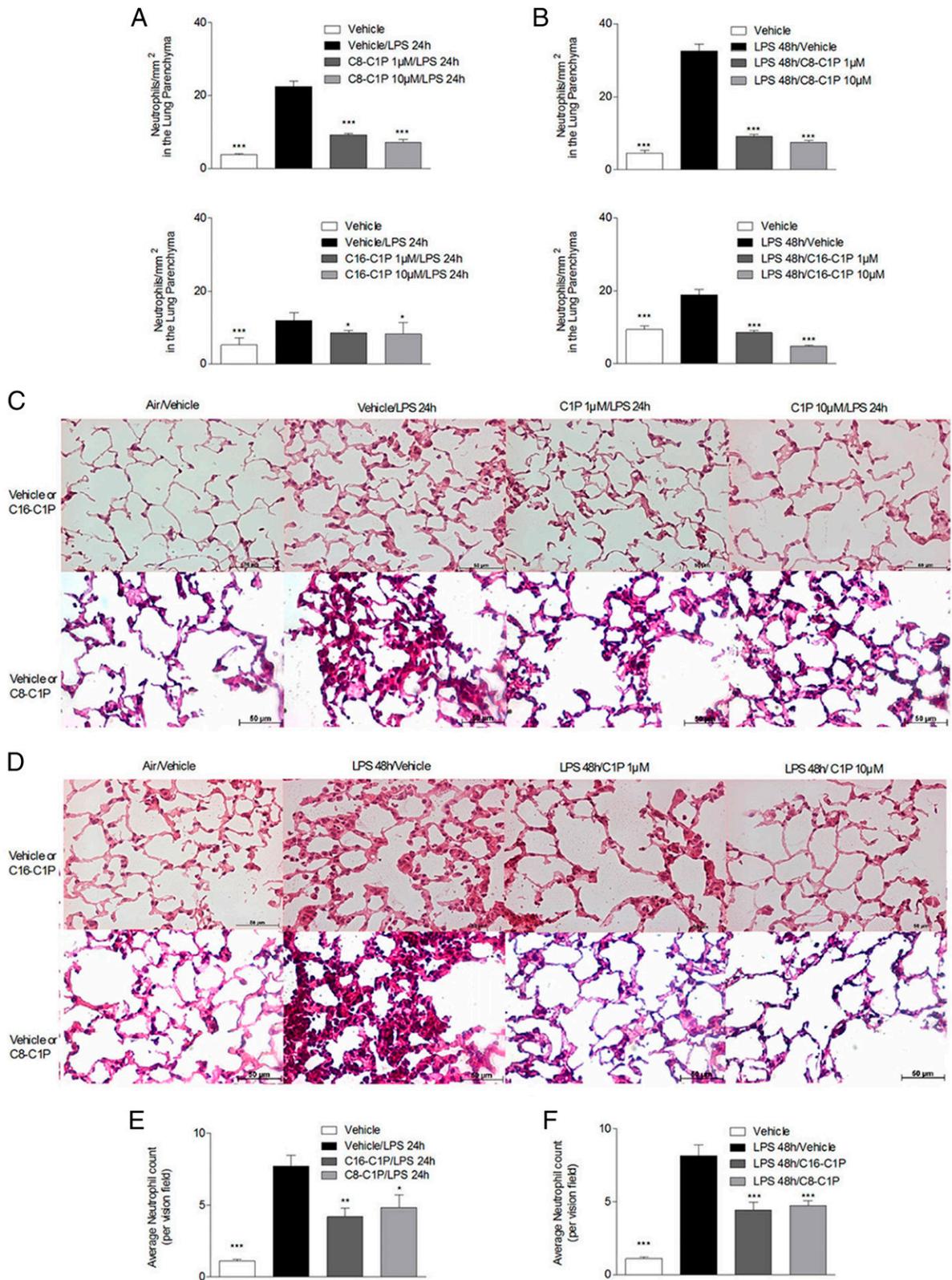
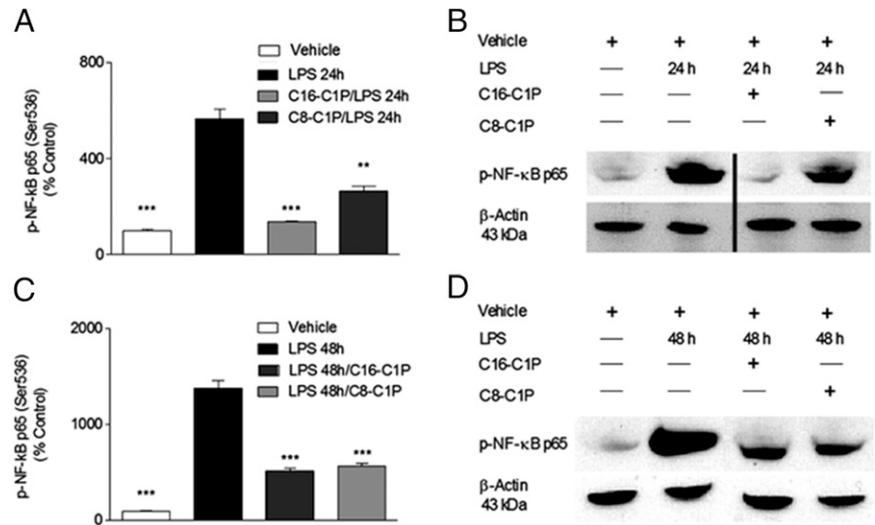


FIGURE 4. Effect of C1P on neutrophil recruitment in ALI. C8-C1P (upper panel) or C16-C1P treatment (lower panel) (1 and 10 μM) were given 1 h before LPS or 24 h after LPS administration. For the prophylactic study (A), animals were euthanized 24 h after LPS and for therapeutic study 48 h after LPS instillation (B). Histological pictures from stained lungs with H&E: prophylactic treatment (C) with C1P and therapeutic administration (D) of C1P. Scale bars, 50 μm. Fluorescently labeled neutrophils in the lung parenchyma were counted: prophylactic treatment with C1P (1 μM) (E) and therapeutic administration of C1P (1 μM) (F). Values are given as mean ± SEM. n = 5 mice in each group. *p < 0.05, **p < 0.01, ***p < 0.001 versus vehicle/LPS 24 h or versus vehicle/LPS 48 h.

FIGURE 5. Prophylactic (A) and therapeutic (C) C1P administration reduced NF- κ B p65 protein expression and activation in lung tissue. C1P (1 μ M) was given before or 24 h after LPS administration. Animals were euthanized 24 or 48 h after LPS administration, respectively. The densitometric analysis of phospho-NF- κ B p65 was performed using ImageJ. Western blotting analysis of the total extract (50 μ g) using phospho-NF- κ B p65 Ab. Western blot of the NF- κ B p65 phosphorylation (upper blot) is shown (B and D). The protein loading control was monitored by staining the same membrane with β -actin (lower blot) (B and D). The black lines indicate where parts of the image were joined. Values are given as mean \pm SEM. $n = 5$ mice in each group. $**p < 0.05$, $***p < 0.001$ versus vehicle/LPS 24 h or versus vehicle/LPS 48 h.



Furthermore, the anti-inflammatory effect of C1P seems strongly influenced by the used C1P concentration. In our previous study, we already described only an anti-inflammatory effect until a C1P concentration of 10 μ M (51), which is in line with the results of Hankins et al. (18). Often a proinflammatory effect of C1P (14, 52) is described either with higher C1P concentrations or different solvent (53, 54). Further studies could clarify the full potential of the C1P concentration regarding the interaction with the cells and the role as exogenous or endogenous C1P compound.

A cardinal feature of LPS-induced ALI is the presence of vascular leakage, leading to the development of pulmonary edema

(20, 26). A significant improvement in lung edema was observed by pretreatment and posttreatment of animals with C1P. The reduction in lung edema might be related to the inhibition of neutrophil recruitment, because neutrophils are considered as the primary cellular effectors of alveolar-capillary damage in ALI. Notably, the potent anti-inflammatory capacity of C1P in ALI is further supported by the fact that this effect was still observed in mice with established LPS-induced lung inflammation.

The pathogenesis of ALI is complex and implies various signal transduction processes. Particular attention has been given to the NF- κ B pathway, which regulates the expression of genes encoding

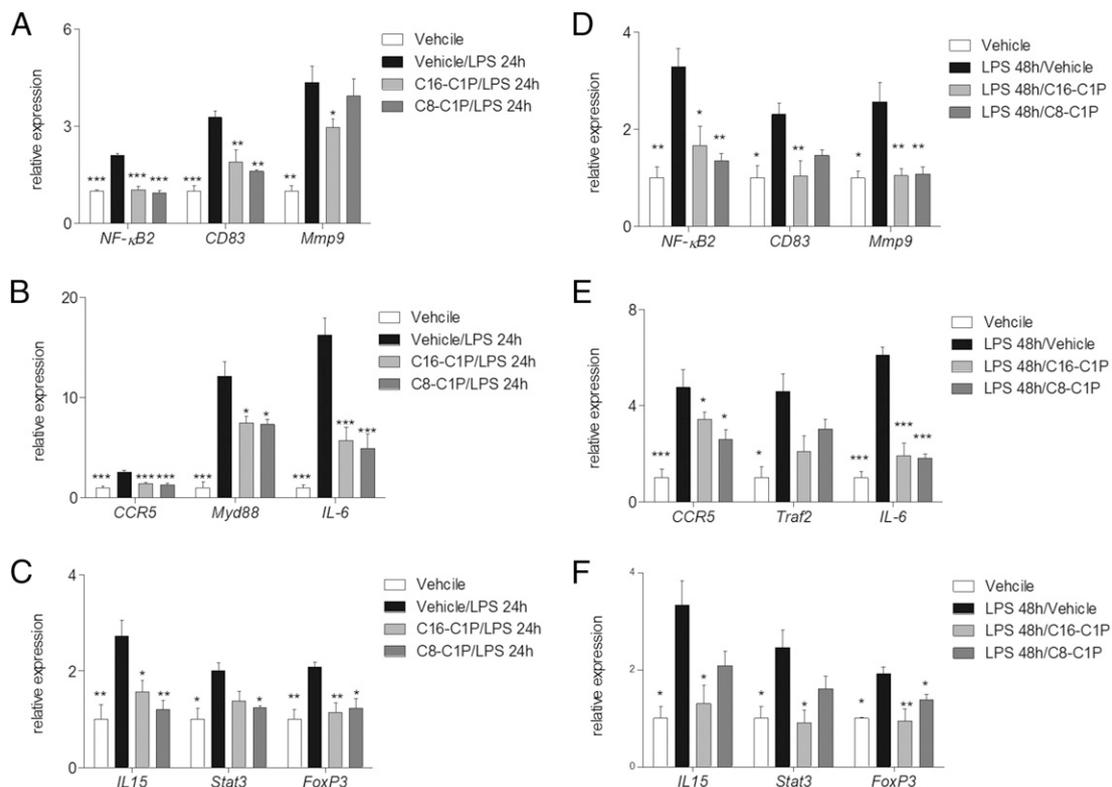


FIGURE 6. C1P attenuated NF- κ B mRNA expression and NF- κ B-related genes in lung tissue of mice. The effect of prophylactic (A–C) and therapeutic (D–F) C1P (10 μ M) treatment in LPS-induced acute lung inflammation on NF- κ B2 and NF- κ B target genes are shown. qPCR was performed for NF- κ B2, MMP-9, and CD83 molecule (CD83) (A and D), CCR5 and IL-6 (B and E), Myd88 (B) and Traf2 (E), IL-15, STAT3, and Foxp3 (C and F). β_2 -MICROGLOBULIN and GAPDH were used as reference genes. Values are mean \pm SEM. Values are means \pm SEMs of at least two biological replicates. $n = 4$ –5 mice in each group of two technical replicates. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ versus vehicle/LPS 24 h or versus LPS 48 h/vehicle.

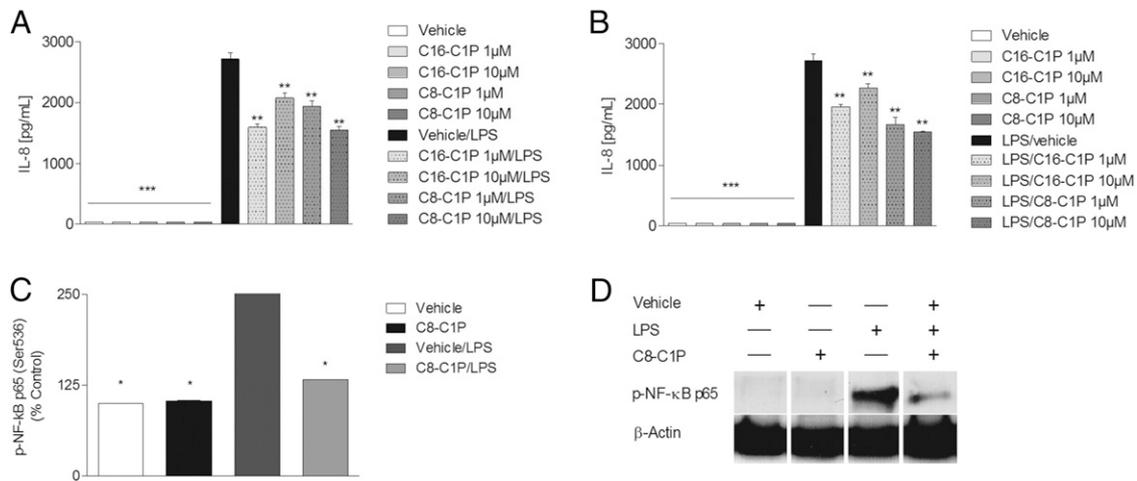


FIGURE 7. C1P administration reduced IL-8 release and NF- κ B expression/activity in human neutrophils. Blood neutrophils (2×10^6 /well) were stimulated with C1P (10 μ M) 1 h before (**A**) and 1 h after (**B**) LPS stimulation (1.5 μ g/ml) for 7 h. Then the medium was collected for analysis of IL-8 levels by ELISA (A and B). For Western blot analysis, C1P (10 μ M) was administered for 60 min to human neutrophils. Afterward, 5×10^6 cells were stimulated with LPS 15 min to evaluate NF- κ B p65 phosphorylation by Western blotting. The densitometric analysis (**C**) was performed using ImageJ. * $p < 0.05$ versus vehicle/LPS. Representative blotting of neutrophils stimulated with C1P (10 μ M) and LPS are shown in (**D**). The protein loading control was monitored by staining the same membrane with β -actin (*lower panel*). Values are given as mean \pm SEM. ** $p < 0.01$, *** $p < 0.001$ versus vehicle/LPS or LPS/vehicle.

mediators involved in inflammatory lung process (55, 56). Increased nuclear translocation of NF- κ B in the lungs of patients with ALI has been reported to correlate with disease severity and outcome (19, 36, 55–57). Moreover, inhibition of NF- κ B activation leads to reduction of acute lung inflammation in experimental models of ALI (48, 56), pointing out the crucial role of the NF- κ B pathway in the pathogenesis of ALI. Furthermore, increased NF- κ B activation in neutrophils of ALI patients showed an important correlation with impaired outcomes of the disease, particularly with diminished time in the ventilator postintubation and increased survival in critically ill patients with ALI (56). Interestingly, we observed that C1P was able to attenuate LPS-evoked *NF- κ B2* mRNA expression in the lung tissue of animals. In addition, we observed an inhibitory effect of C1P on *NF- κ B*-related genes such as *CCR5*, *Mmp9*, *CD83*, *Myd88*, *Traf2*, *IL-6*, *IL-15*, *STAT3*, and *Foxp3*, which are implied in the inflammatory process. Moreover, Western blot assays of NF- κ B phosphorylation (phospho-NF- κ B p65) encourage our finding that C1P affects the LPS-induced NF- κ B signaling pathway in the lung of mice. Similarly, Hankins et al. (18) have shown the inhibitory effect of C1P (10 μ M) on LPS-triggered NF- κ B activation and cytokine release in human embryonic kidney cells. Contrary to LPS stimulation, others reported that higher concentration of C1P (20 μ M) activates NF- κ B in the alveolar rat macrophage cell line NR8383 (52) and in resting cells of the murine macrophage cell line J774A.1 (14). These findings support the hypothesis of Hankins et al. that the inhibition of NF- κ B by exogenous C1P is dose dependent and specific to TLR4.

Furthermore, to better clarify the role of C1P, cell culture experiments with primary human neutrophils were performed. C1P was able to inhibit LPS-triggered NF- κ B p65 phosphorylation in the prophylactic and the therapeutic model, which was also associated with reduction in LPS-induced IL-8 production by these cells.

Although the role of S1P receptors in the pathogenesis of inflammatory disorders has been extensively studied (2, 4, 12), the knowledge about a possible receptor of C1P and its interaction is hardly described. C1P might exert its anti-inflammatory capacity by suppressing the activation of the NF- κ B pathway. We support the argument of Hankins et al. (7, 18, 58) that fluctuations in C1P levels determine its proinflammatory or anti-inflammatory effects,

and further studies are required to clarify the interaction of C1P with the plasma membrane or possible delineated receptors, as well as the effect of C1P in specific cell types. C1P can act either as an intracellular second messenger (10) or as an extracellular mediator binding to functionally identified, but still not cloned, specific G protein-coupled receptor upon secretion to the extracellular milieu (11). Further studies could focus especially on this subject to fully understand the interaction of C1P and its molecular mechanism.

Therefore, we conclude that C1P acts as an important anti-inflammatory agent in LPS-induced acute lung inflammation. Major characteristic parameters of ALI, such as neutrophil activation, proinflammatory cytokines, and vascular plasma leakage are diminished. Mechanistically we showed that C1P attenuates NF- κ B activation in human neutrophils and the expression of *NF- κ B*-related genes in the lungs of mice. Thus, C1P and its analogs offer novel therapeutic targets for the treatment of ALI.

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Disclosures

The authors have no financial conflicts of interest.

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