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Inhibition of the MAP kinase ERK protects from lipopolysaccharide-induced lung injury

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ABSTRACT

The pathogenesis of chronic obstructive pulmonary disease (COPD) is characterized by pulmonary inflammation associated with lung neutrophilia and elevated levels of pro-inflammatory mediators in the bronchoalveolar lavage fluid or sputum of patients. Recent findings revealed that mitogen-activated protein kinase (MAPK) signaling cascade is involved in the inflammatory response of lung injury. In the present study we could elucidate the role of extracellular signal-related MAPK in the murine model of LPS-induced acute lung injury by using U0126, a specific inhibitor of MEK1/2, upstream kinases of ERK. Phosphorylation of ERK was inhibited by U0126 in vivo as well as in vitro. In freshly isolated human peripheral blood mononuclear cells U0126 dose-dependently blocked the release of IL-2 and TNF-α. For in vivo studies mice were exposed to aerosolized LPS to induce an acute lung injury mimicking some aspects of COPD. This led to a recruitment of neutrophils to the lung and to the release of pro-inflammatory cytokines into bronchoalveolar lavage. Pretreatment of mice with U0126 significantly reduced lung neutrophilia and diminished levels of TNF-α and chemotactic MIP-2 and KC in bronchoalveolar fluid. U0126 also decreased albumin levels in BAL fluid, a marker of vascular leakage. Histological examination of lung tissues revealed that ERK MAPK inhibition using U0126 efficiently attenuated LPS-induced pulmonary inflammatory responses. These data suggest that ERK signaling plays an important role in acute lung injury and pharmacologic inhibition of ERK provides a promising new therapeutic strategy for lung inflammatory diseases and in particular COPD.

1. Introduction

Chronic obstructive pulmonary disease (COPD) is characterized by airflow obstruction that is not fully reversible. It is a major global health problem with growing incidence, which places an increasing burden on health services in industrialized and developing countries [1]. The term COPD encompasses emphysema, chronic obstructive bronchitis and a chronic inflammation in the smaller airways and lung parenchyma [2,3]. The local inflammation observed in COPD patients is predominated by neutrophilic granulocytes [4]. Furthermore increased numbers of macrophages and T-cells, predominantly CD8+ T-cells, are found in the alveolar walls of COPD patients [5].

Cigarette smoke is the major risk factor for the development of COPD, but also exposure to air pollution and genetic factors play an important role [2]. Tobacco and cigarette smoke contain high levels of lipopolysaccharide (LPS), a cell wall component of gram-negative bacteria, which is a potent inflammatory stimulus [6]. Inhalation of LPS causes acute and chronic inflammation in murine airways [7], which is accompanied by recruitment of neutrophils to the lung and increased levels of pro-inflammatory cytokines and chemokines in the lung. Acute or chronic inhalation of LPS mimics some relevant features of COPD in mice and serves as a model for preclinical analysis of possible drug candidates [8].

LPS induced lung injury is mediated through Toll-like-receptor 4 (TLR4) and CD14 ligation, that leads to the activation of mitogen-activated protein kinase (MAPK) signaling cascades, which ultimately results in the secretion of tumor necrosis factor α (TNF-α) and other pro-inflammatory cytokines [9,10]. The MAPK families are ubiquitous and highly conserved serine-threonine

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Abbreviations: COPD, chronic obstructive pulmonary disease; MAPKK, mitogen-activated protein kinase kinase; MAPKKK, mitogen-activated protein kinase kinase kinase; LPS, lipopolysaccharide; ERK, extracellular signal-related kinase; MEK, extracellular signal-regulated kinase kinase; TNF, tumor necrosis factor; PBMC, peripheral blood mononuclear cells; MIP, macrophage inflammatory protein; KC, keratinocyte-derived chemokine; BAL, bronchoalveolar lavage; MPO, myeloperoxidase.

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kinases, including p38MAPK, extracellular signal-related kinase (ERK) and c-Jun N-terminal kinase (JNK). The signaling cascade is initiated by three-tired sequential phosphorylation steps (MAPKKK, MAPKK, MAPK). MEK1 and MEK2 are upstream kinases (MAPKK) of ERK1 and ERK2. Recently a number of studies revealed that p38 MAPK is an important enzyme for the pathogenesis of LPS-induced lung injury. Compound 37, a specific p38 α,β MAPK inhibitor, attenuated LPS-induced bronchoconstriction and neutrophil recruitment into the lungs in a dose-dependent manner [11]. It was concluded, that p38 MAPK mediates lung neutrophilia, cytokine and chemokine production and bronchoconstriction [11,12].

It has been shown that ERK1/2 pathway plays a pivotal role in IL-13-induced asthma-like lung inflammation [13]. We hypothesized that the ERK1/2 pathway also plays an important role in LPS-mediated pulmonary inflammation and that inhibition of ERK signaling pathway may have anti-inflammatory effects. To this end we investigated the impact of U0126, a selective and potent MEK1/2 inhibitor, in the mouse model of acute LPS-induced lung injury and examined wether pharmacologic inhibition of ERK attenuates disease severity.

2. Materials and methods

2.1. Animals

All animal protocols were performed in accordance with the national animal protection rules and permitted by the local governmental authority (Regierung von Mittelfranken, Germany). BALB/c mice 6–8 weeks of age weighing 22–25 g (purchased from Harlan, Borchen, Germany) were used. The animals were fed on a normal standard diet (food pellets purchased from Altromin, Lage, Germany) with tap water ad libitum and were housed in a 12 h light/dark cycle, at 20–21 °C and with 40–60% humidity levels.

2.2. Mouse model of LPS-induced lung inflammation

The animals were treated intraperitoneally with U0126 (30 mg/kg; purchased from Tocris Biosciences, Ellisville, USA), PD98059 (30 mg/kg; Tocris Biosciences, Ellisville, USA) or vehicle (200 μl PBS with 5% DMSO, Invitrogen, Karlsruhe, Germany) 2 h prior to LPS challenge. U0126, PD98059 or vehicle-treated mice were exposed to aerosolized LPS (10 mg; 1.5 mg/ml saline; lipopolysaccharides from *Escherichia coli* Serotype 026:B6, Sigma, St. Louis, USA) using the Buxco-Nebulizer (Buxco, Wilmington, USA). One inhalation cycle encompassed 9.9 min of LPS (25% duty) and followed by 5 min of drying. This was repeated four times. Animals were sacrificed 24 h or at the indicated time point after LPS challenge by putting them into a CO_2 rich atmosphere. Tracheotomy was performed and a 24G cannula (InSyte 24G, BD Biosciences, Heidelberg, Germany) was inserted in the trachea. In each animal bronchoalveolar lavage (BAL) was performed by flushing the airways and lungs (6×0.5 ml) with cold Hank's balanced solution (Gibco, Karlsruhe, Germany) supplemented with Na-EDTA and HEPES (Sigma, Steinheim, Germany). The supernatants of the first lavage were collected and stored at −20 °C for further analysis. The cells of pooled BAL of each animal were sedimented by centrifugation at 300 × *g* for 5 min. The cell pellets were re-suspended in 2 ml RPMI-1640 (Invitrogen, Karlsruhe, Germany) and total BAL cell counts of each animal were determined using a haemocytometer (Sysmex microcellcounter F300, Norderstedt, Germany). For cytological examination, cytospin slides were prepared using a cytospin centrifuge (Thermo Shandon, Frankfurt, Germany) and stained with Diff-Quick (Dade-Behring, Marburg, Germany). Differential cell count was performed with at least 400 cells/slide.

2.3. Cytokine and chemokine measurements

Levels of tumor-necrosis factor α (TNF-α), macrophage inflammatory protein 2 (MIP-2) and keratinocyte-derived chemokine (KC) in BAL fluid were measured using a murine TNF-α OptEIA ELISA kit (BD Biosciences, San Diego, USA) and murine MIP-2 and murine KC DuoSet ELISA Development kits (R&D Systems, Minneapolis, USA). Levels of TNF-α and interleukin 2 (IL-2) in supernatants of stimulated PBMC were determined using a human TNF-α OptEIA ELISA Set and a human IL-2 OptEIA ELISA kit (BD Biosciences, San Diego, USA). Cytokine and chemokine levels were assayed according to the manufacturer's recommendations.

2.4. Phospho-p44/42 MAPK and phospho-p38 MAPK sandwich ELISA

Mice were treated intraperitoneally with U0126 (30 mg/kg) or vehicle 2 h prior to LPS challenge. Two hours later mice were sacrificed the lungs were removed and stored in liquid nitrogen. After homogenization by ultra turrax the lysate was microcentrifuged and the supernatant was used for detection of pERK and pp38 (PathScan Phospho-p44/42 MAPK (Thr202/Tyr204) Sandwich ELISA Kit and PathScan Phospho p38α MAPK (Thr180/Tyr182) Sandwich ELISA Kit (Cell Signaling, Danvers, USA)) according to the manufacturers recommendations. Absorbance at 450 nm is proportional to the quantity of p44/42 MAPK phosphorylated at Thr202/Tyr204 and p38 MAPK phosphorylated at Thr180/Tyr182.

2.5. Lung histological examination

Lungs were instilled intratracheally with 15 ml 4% buffered formaldehyde (Roth, Karlsruhe, Germany) using a syringe pump (sp200i, WPI, Berlin, Germany) and fixed overnight in formaldehyde. The tissues were embedded in paraffin and 4 μm slices were cut. To examine bronchial inflammation sections were stained with conventional hematoxylin and eosin (Roth, Karlsruhe, Germany). Bronchial inflammation was determined using a semiquantitative score as described by Myou et al. [14]. The severity of inflammation was graded in five categories; 0, normal lung structure; 1, few inflammatory cells; 2, a ring of inflammatory cells 1 cell layer deep; 3, a ring of inflammatory cells 2–4 cells deep; 4, a ring of inflammatory cells of >4 cells deep. The score was determined in a blinded manner.

2.6. Myeloperoxidase assay

Determination of myeloperoxidase activity was adapted from Matos et al. [15]. The lungs were flash frozen in liquid nitrogen immediately after removing and stored at −80 °C until examination. The frozen lungs were homogenized with an ultra turrax in 1 ml Na_2PO_4 buffer (0.05 M, pH5.4) and centrifuged for 10 min at 20,000 × *g* at 4 °C. The supernatants were discarded. The pellet was resuspended in 1 ml Na_2PO_4 buffer containing 0.5% hexadecyltrimethylammonium bromide (Sigma–Aldrich, St. Louis, USA) and homogenized. This was followed by three freeze-thaw cycles using liquid nitrogen. After centrifugation at 20,000 × *g* for 10 min at 4 °C the supernatants were collected. Tetramethylbenzidine (TMB) and hydrogen peroxide (TMB Substrate Reagent Set, BD Biosciences, San Diego, USA) were added to the supernatants and the reaction was stopped after 5 min with 1 M H_3PO_4. The activity of MPO was quantified by measuring absorbance at 450 nm.

2.7. Vascular leakage

The vascular leakage was determined by measurement of total albumin levels in supernatants of bronchoalveolar lavage using a Bradford-Assay (Bio-Rad, München, Germany).

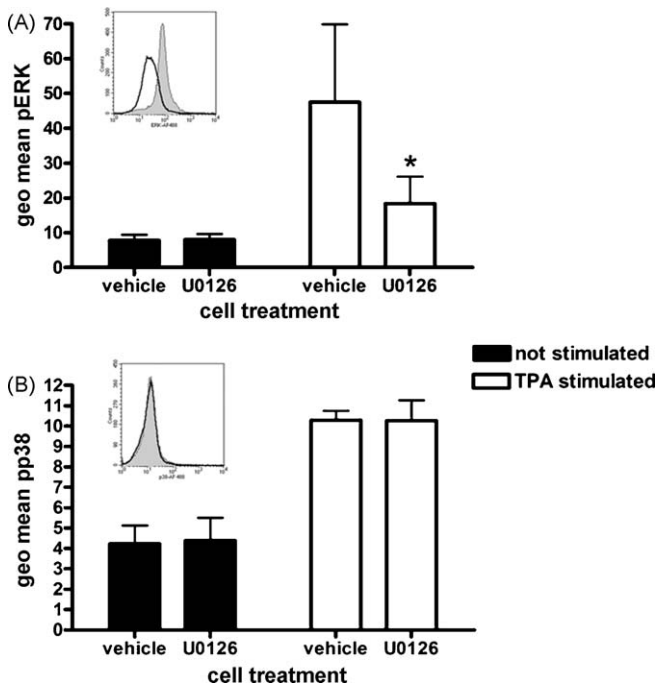

Fig. 1. U0126 inhibits phosphorylation of ERK in stimulated human PBMC specifically. Peripheral blood mononuclear cells from healthy donors were isolated by density gradient centrifugation, preincubated with U0126 (1 μM) and stimulated with TPA. 15 min after stimulation cells were fixed, permeabilized, stained intracellular with phosphospecific antibodies anti-pERK (A) and anti-pp38 (B) and analyzed by flow cytometry. Inserts show representative FACS plots of TPA stimulated and vehicle treated (shaded in gray) or U0126 treated (black line) cells. Data are expressed as geometric means of fluorescence intensity ± SEM ($n = 4$). $^*p < 0.05$ compared to vehicle treated cells.

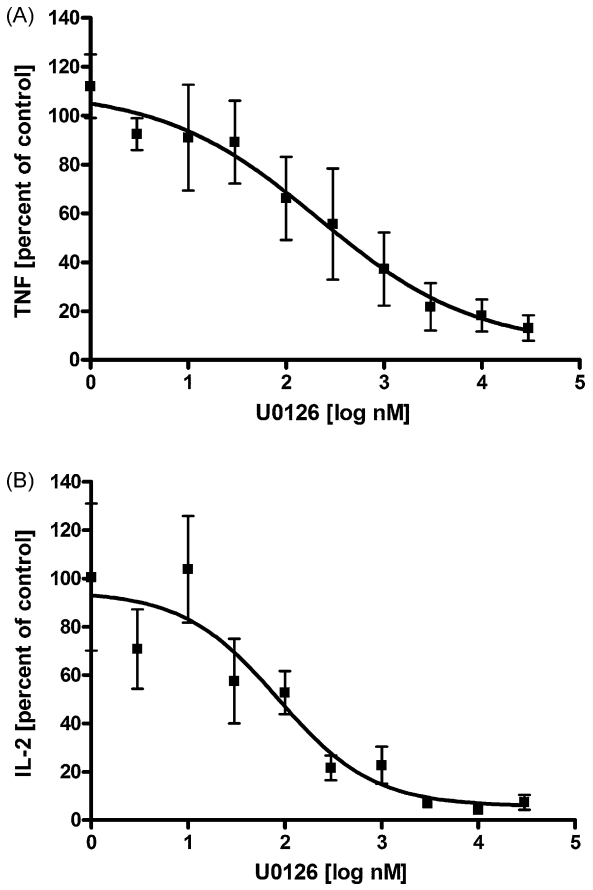

Fig. 2. U0126 dose-dependently inhibits cytokine release from stimulated human PBMC. Isolated human PBMCs were preincubated with increasing concentrations of U0126 and stimulated with LPS for measurement of TNF-α-levels (A) or anti-CD3/CD28-beads for measurement of IL-2 levels (B). 24 h later supernatants were collected and ELISA analysis was performed. Data were normalized to vehicle treated cells and expressed as mean ± SEM ($n = 3$ different donors).

2.8. Isolation and treatment of peripheral blood mononuclear cells (PBMC)

PBMC were isolated from EDTA-anticoagulated venous blood from healthy human volunteers by using density-gradient centrifugation over Ficoll-Histopaque (Sigma, St. Louis, USA). In brief, blood samples were diluted 1:1 with Hanks balanced salt solution and carefully layered over Histopaque. After centrifugation at 742 × *g* for 20 min the PBMC rich film above the Histopaque was collected and washed twice with Hanks buffer. The number of PBMC was determined using a haemocytometer and afterwards re-suspended at a density of 1×10^6 cells/ml in RPMI 1640. The cells were plated in 24-well-plates and cultured for 24 h at 37 °C in a 5% CO_2 atmosphere.

For flow cytometry analysis cells were preincubated with U0126 (1 μM) for 30 min and then stimulated with TPA (Phorbol-12-myristate-13-acetate) (0.1 μg/ml) (Sigma, St. Louis USA). 15 min later cells were fixed with 1.5% formaldehyde and then stained intracellular with specific antibodies.

For cytokine measurements cells were preincubated with various concentrations of U0126 (1 μM-30 mM) for 30 min. Cells were stimulated with LPS (1 mg/ml) for TNF-α analysis or anti-CD3/CD28-beads (2 μl/well) (Invitrogen Dynal, Oslo, Norway) for measurement of IL-2 levels. 24 h later supernatants were collected and stored at −80 °C until analysis.

2.9. Flow cytometric analysis (FACS)

PBMC were fixed with 1.5% formaldehyde and permeabilized with ice-cold 100% methanol. Intracellular staining was performed after Fc-block with Phosflow antibodies: ERK 1/2 AF488, p38 MAPK AF488 and isotype control antibody (BD Biosciences, San Diego, USA). Cells were analyzed using a FACScan-flow cytometer and CellQuest Software (BD Biosciences). Geometrical mean and mean were used for analysis.

2.10. Statistical analysis

GraphPad Prism (Version 5) software was used for analysis of data. Data are presented as means ± SEM. Statistical significance was determined by the Students unpaired *t*-test for comparison of two groups. *P*-values of $p < 0.05$ were considered statistically significant.

3. Results

3.1. U0126 inhibits ERK activation in human primary cells

The specificity of U0126 was determined by analyzing phosphorylated MAPK in stimulated human PBMC using flow cytometry. Stimulation of cells with LPS yielded only a weak activation under these conditions. Due to the low signal to noise ratio, we decided to use the robust and strong stimulus TPA for pharmacological characterization of U0126 in vitro. The phosphorylated forms of ERK and p38 were stained intracellularly with phosphospecific antibodies. Stimulation with TPA increased the levels of phosphorylated ERK as well as of phosphorylated p38 (Fig. 1). Pretreatment of human PBMC with U0126 (1 μM) significantly reduced levels of phosphorylated ERK ($p = 0.049$) (Fig. 1A). In contrast, activation of p38 was not affected by cell treatment with U0126 (1 μM) (Fig. 1B). Cell viability was analysed by the WST assay. No cytotoxicity was

detected up to the highest concentration of U0126 (30 μM) (data not shown).

3.2. Dose dependent inhibition of cytokine induction in stimulated PBMC by U0126

Next, we analyzed whether the specific inhibition of pERK by U0126 affects cytokine release. Previously it has been shown that LPS activates ERK in macrophage cell lines and human monocytes [16] and that CD3/CD28 stimulation activates MAPKs in T-cells [17]. We stimulated human monocytes with LPS and lymphocytes with anti-CD3/CD28 beads in absence or presence of various concentrations of U0126. This compound dose-dependently inhibited LPS induced TNF-α with an IC_{50} of 0.21 μM (Fig. 2A). Likewise IL-2 release in stimulated lymphocytes was dose-dependently inhibited by U0126 with an IC_{50} of 0.9 μM (Fig. 2B).

3.3. Effects of U0126 on LPS-induced lung injury in vivo

To evaluate the effects of MEK1/2 inhibition in vivo the model of acute LPS induced lung injury was used. BAL fluid was collected 24 h after LPS aerosol challenge and total and differential cell counts were determined. LPS challenge led to massive influx (20-fold increase compared to negative control) of inflammatory cells into the airways. Pretreatment of mice with U0126 reduced total cell numbers recovered in BAL fluid significantly ($p = 0.009$; Fig. 3A). The increased cell infiltration consisted primarily of neutrophils. U0126 inhibited neutrophil numbers recovered in BAL fluid significantly as compared to vehicle treated mice ($p < 0.001$) (Fig. 3B). In addition decreases in macrophage ($p = 0.046$) and lymphocyte ($p = 0.026$) populations were detected as well (Fig. 3B). The reduced neutrophil numbers among U0126-treated mice were consistent with decreased MPO activity in lung tissue ($p = 0.044$) (Fig. 3C). Furthermore U0126 administration caused significant reduced albumin levels ($p = 0.047$) in BAL fluid supernatants as a marker for decreased lung permeability and inflammation (Fig. 3D).

3.4. Effect of U0126 on ERK activation in vivo

In order to analyze the pharmacodynamic action of U0126 we assessed levels of phosphorylated ERK in lung tissue. LPS challenge caused substantially increased phosphorylation of MAPK (Fig. 4A). Lungs obtained from U0126–treated mice showed significantly reduced phosphorylated ERK level ($p = 0.04$) indicating the inhibition of phosphorylated ERK by U0126 in the lungs of LPS treated mice (Fig. 4A). In contrast, phosphorylation of p38 in lung tissue was not affected by U0126. Levels of phosphorylated p38 of lungs obtained from U0126-treated mice were comparable with positive controls (Fig. 4B).

3.5. Effect of U0126 on cytokine and chemokine levels in BAL fluid

It has been shown that MIP-2, KC and TNF-α play a pivotal role in LPS-induced lung injury [18]. To evaluate this in vivo BAL fluid samples were collected 2 h after LPS challenge and levels of these cytokines and chemokines were determined. LPS inhalation induced elevated cytokine release into BAL fluid as compared to

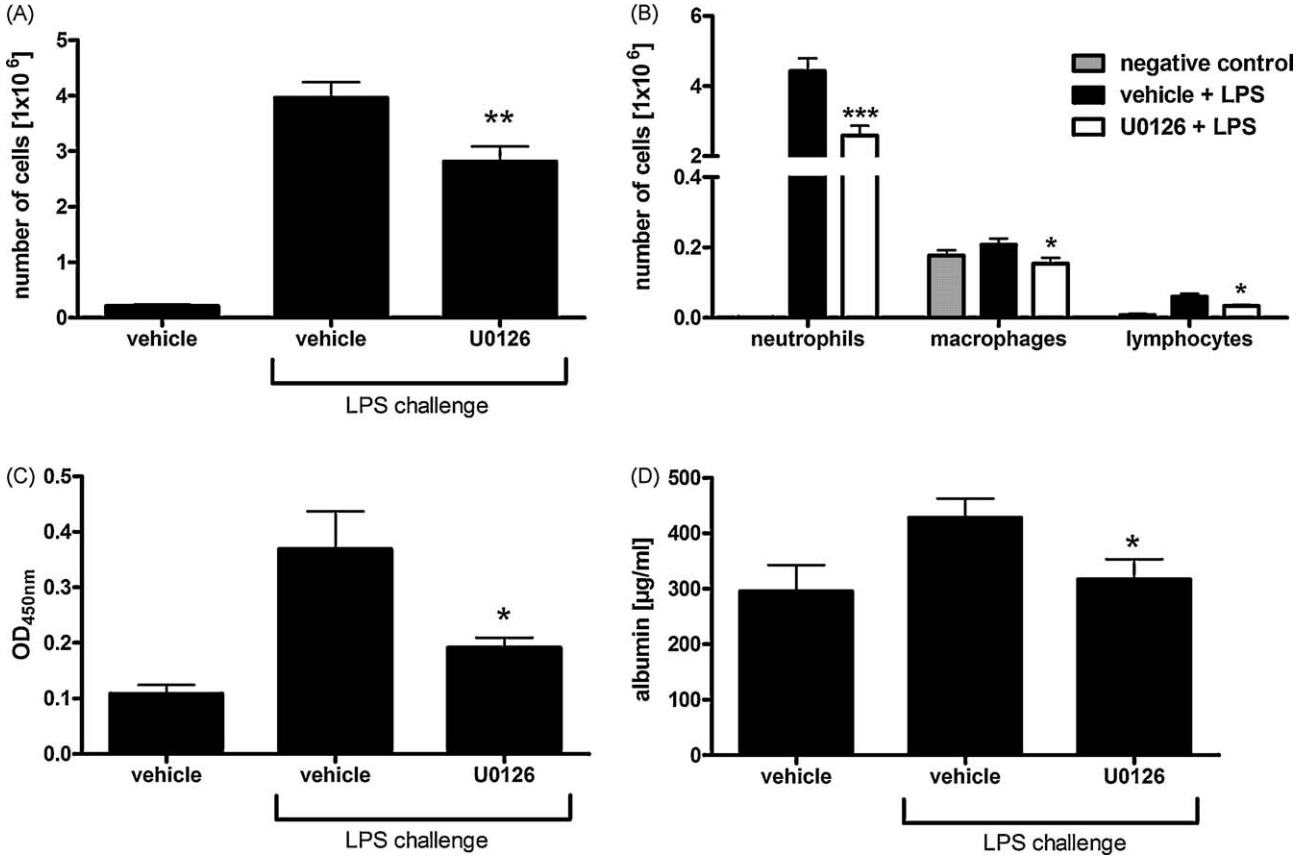

Fig. 3. U0126 reduces LPS-induced cellular infiltration into bronchoalveolar lavage fluid. Mice were treated intraperitoneally with U0126 (30 mg/kg) or vehicle (5%DMSO in PBS) 2 h prior to LPS challenge. 24 h later mice were sacrificed and bronchoalveolar lavage was performed. Total (A) and differential cell counts (B) were performed in bronchoalveolar lavage fluid. Counts of minimum 400 cells/animal were accomplished to identify neutrophils, macrophages and lymphocytes (B). Data are expressed as absolute cell numbers. MPO activity was measured in lung tissue (C). Albumin levels were determined in supernatants of BAL fluid (D). Data are expressed as means ± SEM (A, B, C $n = 10$ mice/group, D $n = 7$). $^{*}p < 0.05$, $^{**}p < 0.01$ as compared to vehicle control.

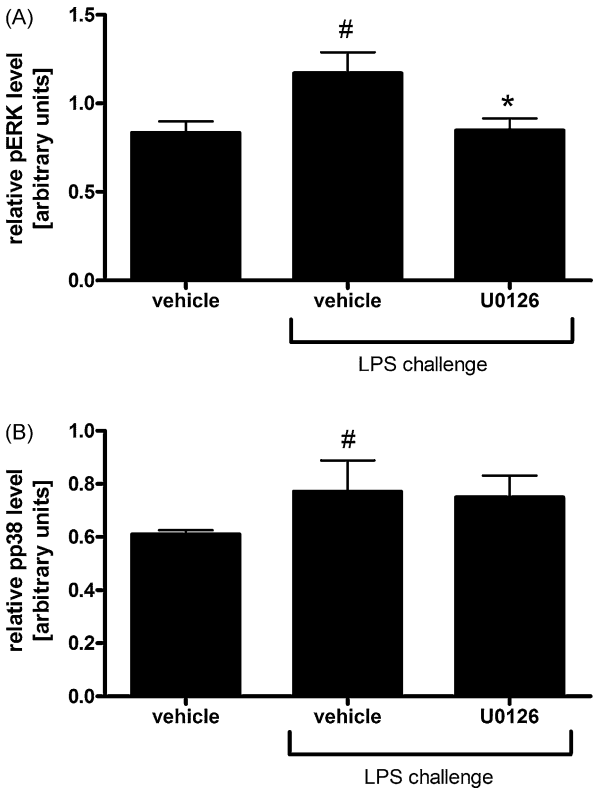

Fig. 4. Inhibition of phosphorylated ERK by U0126 in lung tissue. Mice were treated intraperitoneally with U0126 (30 mg/kg) or vehicle 2 h prior to LPS challenge. 2 h after LPS challenge mice were sacrificed and the lungs were removed. Lung lysates were analyzed for phosphorylated ERK (A) and p38 (B) levels using sandwich ELISA. The absorbance at 450 nm was measured and is shown as optical density ± SEM ($n = 6$). $^{\#}p < 0.05$ as compared to vehicle without LPS stimulation; $^{*}p < 0.05$ as compared to LPS stimulated vehicle control.

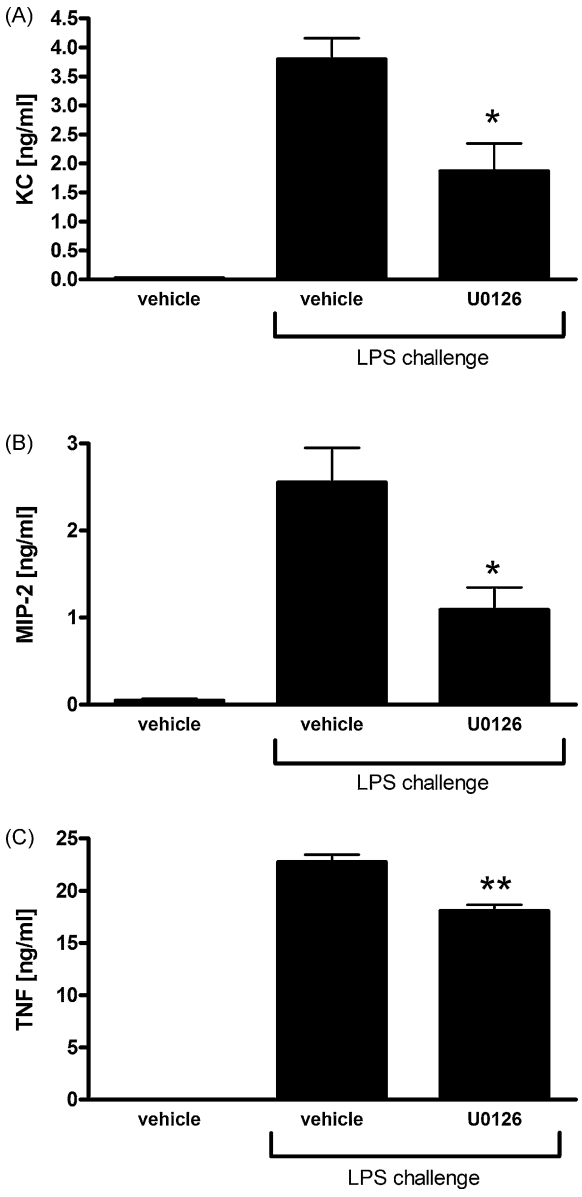

Fig. 5. Effect of U0126 on cytokine and chemokine levels in bronchoalveolar fluid. Animals were treated 2 h prior to LPS challenge intraperitoneally with U0126 (30 mg/kg) or vehicle control. 2 h later bronchoalveolar lavage was performed. The lavage was centrifuged and the supernatants were used for ELISA. Levels of murine KC (A), MIP-2 (B) and TNF-α (C) were assessed by standard sandwich ELISA with a detection limit of 15.6 pg/ml for each cytokine. Data are expressed as means ± SEM ($n = 4$). Statistically significant differences at $^{*}p < 0.05$, $^{**}p < 0.01$, as compared to LPS stimulated vehicle control.

untreated mice. Treatment of mice with the MEK 1/2 inhibitor U0126 prior to LPS challenge led to significantly reduced levels of KC ($p = 0.015$), MIP-2 ($p = 0.049$) and TNF-α ($p = 0.008$) as compared to vehicle treated animals (Fig. 5A–C).

3.6. Effect of U0126 on lung inflammation in vivo

The effect of U0126 on LPS induced lung inflammation was analyzed morphologically by histological analysis. Lung tissue was collected 24 h after LPS challenge. Light microscopy of haematoxylin-eosin stained lung slices revealed marked infiltration of inflammatory cells into peribronchial and perivascular connective tissues after LPS aerosol challenge as compared to naive mice (Fig. 6A, B compared to E, F). Edematous changes of the alveolar walls, swelling of alveolar epithelial cells and massive influx of polymorphonuclear cells were observed. The majority of the infiltrated cells were neutrophils. Administration of U0126 (30 mg/kg) markedly attenuated the neutrophil-rich leukocyte infiltration as compared to vehicle control ($p = 0.007$) (Fig. 6C, D, G).

3.7. Effects of PD98059 on LPS-induced lung injury in vivo

To rule out that the effects of MEK blockade by U0126 are non-specific another structurally unrelated MEK inhibitor was tested. Administration of PD98059 significantly reduced total cell numbers in BAL fluid ($p = 0.029$), lung neutrophilia ($p = 0.025$) and MPO activity in lung tissue ($p = 0.024$) as compared to vehicle controls (Fig. 7).

4. Discussion

The lack of effective pharmacological intervention is still a major problem in chronic inflammatory diseases of the lung, especially COPD. LPS-induced acute lung injury features some relevant characteristics of the disease and therefore could serve as a preliminary model for COPD [8]. Inhibition of MAPK pathways may provide novel anti-inflammatory therapies. There are several reports that the MAPK p38 plays a pivotal role in acute lung injury [11,12]. Moreover evidence has accumulated over the past years that the MAPK ERK is activated by LPS in vitro [16,19]. These observations led us to hypothesize that the ERK1/2 signaling pathway also plays a decisive role in LPS mediated pulmonary inflammation and that inhibition of ERK may have anti-inflammatory effects. To our knowledge, in the present study we have

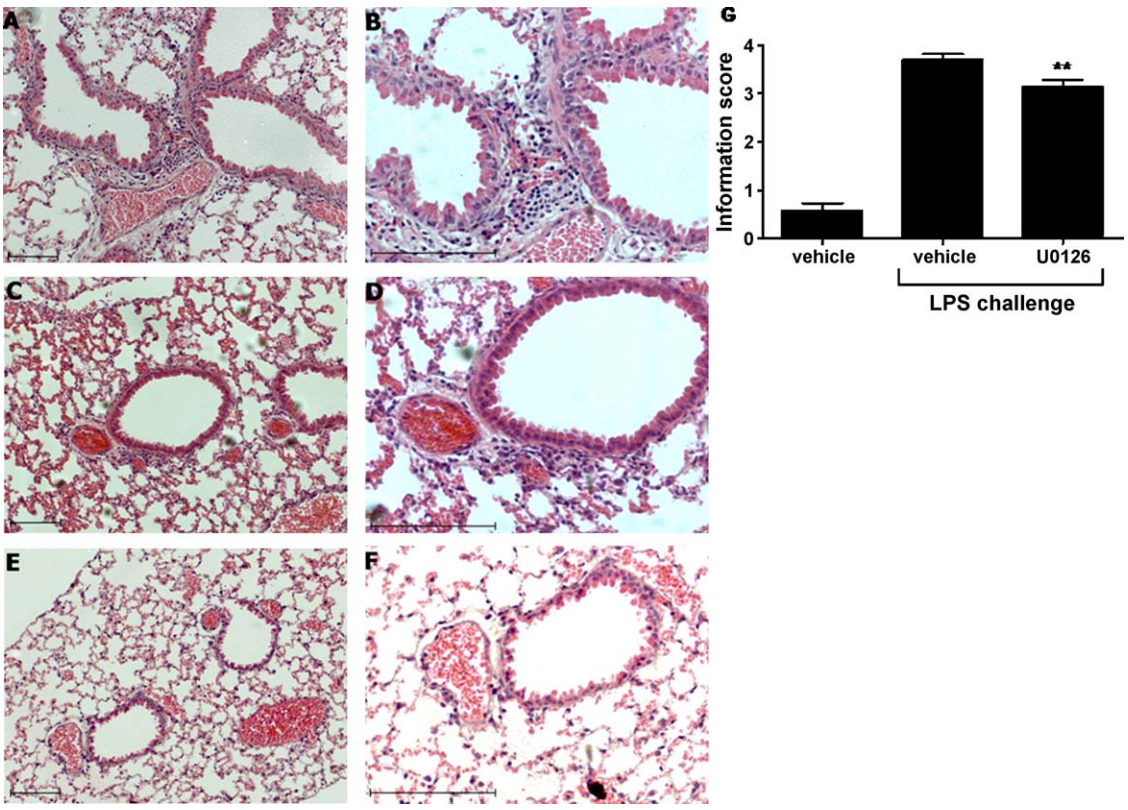

Fig. 6. Effects of U0126 on lung inflammation. Representative lung sections are shown. 24 h after LPS challenge lungs were fixed, embedded in paraffin and cut into 4 μm slices. After H&E staining, histological examination was performed by light microscopy (A, C; E magnification ×200, B, D; F magnification ×400) and inflammation was quantified (G) using a score described by Myou et.al [14]. Lung sections were obtained from vehicle treated mice (A, B), U0126 treated animals (30 mg/kg; C, D) or negative controls (E, F). Scale bars represent 100 μm. Data are presented as means ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$ as compared with positive control.

demonstrated for the first time that pharmacological inhibition of the ERK pathway using U0126, a selective and potent MEK1/2 inhibitor, attenuated inhaled LPS-induced lung injury in an animal model of acute lung injury. These results were corroborated by showing that an unrelated inhibitor of the ERK cascade inhibited acute lung injury as well.

U0126 is a small molecule compound that was initially identified as an inhibitor of AP-1 transactivation in a cell-based reporter assay [20]. U0126 directly inhibits MEK1/2 through inhibition of the catalytic activity of the active enzyme. We have demonstrated that U0126 is specific inhibitor of ERK phosphorylation in vitro as well as in vivo. TNF-α production in LPS-treated monocytes has been shown to be MAPK dependent, which can be blocked by ERK and p38 inhibitors [21]. We observed similar results in freshly isolated human PBMC. The production of TNF-α was dose-dependently inhibited by U0126 via ERK blockade. The observed IC_{50} value of 0.21 μM corresponds to findings in a previous study [16]. Recent studies demonstrated that CD3/CD28 ligation in T-cells activates ERK and up-regulates IL-2 production [22]. In agreement with a previous report U0126 [17] was capable of reducing IL-2 production dose-dependently (IC_{50} = 0.9 μM) in stimulated human PBMC.

Duan et al. previously showed that U0126 (using a dose of 30 mg/kg) effectively reduced lung inflammation, mucus plugging and pulmonary eosinophilia in an asthma mouse model [23]. To investigate the inhibitory effects of U0126 in vivo we used a murine model of a COPD-like acute disease, wherein aerosolized LPS induces an acute lung injury. In lung tissue U0126 specifically blocked phosphorylation of ERK whereas activation of p38 was not affected demonstrating the specificity of the inhibitor used.

Consistent with previous studies exposure to LPS led to massive influx of inflammatory cells, primarily neutrophils peaking 24 h after LPS exposure [3]. Neutrophils undergo directed migration to an inflamed area along a gradient of chemokines. LPS is known to induce the production of several inflammatory and chemotactic cytokines [24]. We observed that concurrently with the massive influx of neutrophils and mononuclear cells, LPS exposure led to a massive increase of the levels of the pro-inflammatory cytokine TNF-α and the neutrophil chemotactic chemokines KC and MIP-2 in BAL fluid. This mimics the clinical situation, whereas neutrophilia and elevated cytokine levels in sputum and BAL fluid are typical clinical findings among patients with acute lung injury. Administration of U0126 inhibited effectively inflammatory cell influx in the BAL fluid of mice exposed to LPS. Since administration of another structurally unrelated MEK inhibitor PD98059 was efficiently in reducing lung neutrophilia as well, non-specific effects of U0126 can be ruled out. Furthermore, inhibition of ERK MAPK resulted in decreased production of the pro-inflammatory cytokine TNF and the neutrophil chemoattractants KC and MIP-2. It is tempting to speculate that inhibition of these chemokines by the MEK1/2 inhibitor may contribute to the decreased lung neutrophilia.

Concomitant with diminished lung neutrophilia U0126 led to reduced MPO activity in LPS challenged mice. A number of reports revealed an important role for MPO in the microbicidal activity of neutrophils and that it is released during neutrophil activation [25,26]. Our finding that inhibition of ERK resulted in reduced both lung neutrophilia and MPO activity support the efficiency of this inhibitor.

An increase of alveolar epithelial permeability is a characteristic feature of several chronic lung diseases like asthma or COPD. It is well known that LPS injures lung endothelial and epithelial cells and enhances lung permeability [27]. Recently it has been shown that acute lung injury can be attenuated by reduction of lung hyperpermeability [28]. In this study we observed that endotoxin inhalation led to elevated albumin levels in bronchoalveolar

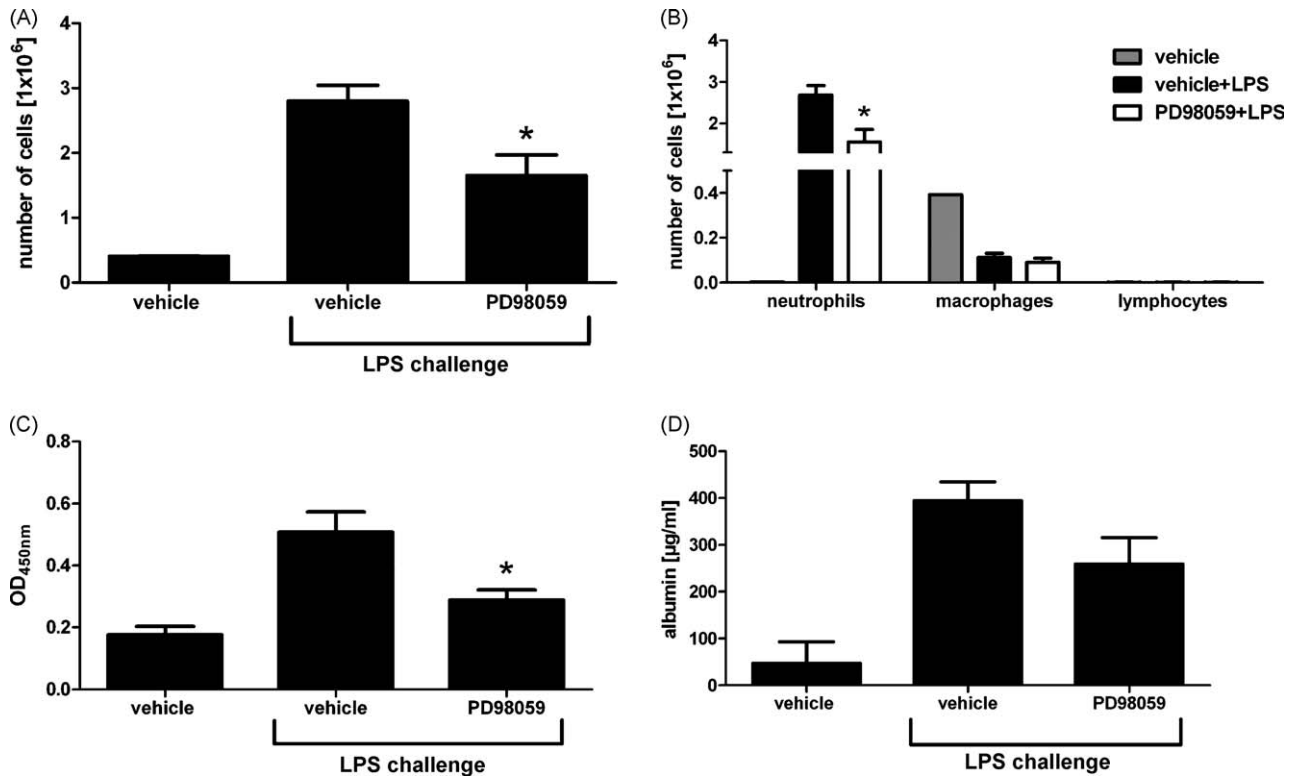

Fig. 7. PD98059 reduces LPS-induced cellular infiltration into bronchoalveolar lavage fluid. Mice were treated intraperitoneally with PD98059 (30 mg/kg) or vehicle (5%DMSO in PBS) 2 h prior to LPS challenge. 24 h later mice were sacrificed and bronchoalveolar lavage was performed. Total (A) and differential cell counts (B) were performed in bronchoalveolar lavage fluid. Counts of minimum 400 cells/animal were accomplished to identify neutrophils, macrophages and lymphocytes (B). Data are expressed as absolute cell numbers. MPO activity was measured in lung tissue (C). Albumin levels were determined in supernatants of BAL fluid (D). Data are expressed as mean ± SEM (n = 6 mice/group). $^*p < 0.05$ as compared to vehicle control.

lavage, which is marker of increased lung epithelial permeability. U0126 was highly efficient in reducing LPS induced albumin levels in BAL fluid. Thus the reduction of lung hyperpermeability may contribute to therapeutic effects of U0126 on acute lung injury.

Emphysematous alterations of alveolar walls and destruction of lung parenchyma accompanied by massive infiltration of inflammatory cells are hallmarks of COPD. Long term LPS exposure leads to a prominent infiltration of macrophages and CD8+ lymphocytes to peripheral airways [29]. In contrast short term exposure to LPS entails rapid influx of neutrophils that peaks 24 h after challenge and diminishes near baseline 72 h later [30]. Similar results were obtained in our study, where histological analysis of the lung revealed a massive infiltration of neutrophils. Blocking ERK by U0126 successfully abated lung inflammation and reduced tissue neutrophilia. This corroborates our findings in BAL and indicates that pulmonary neutrophil infiltration is ERK dependent.

In conclusion, the ERK signaling pathway is crucial for LPS-induced lung injury. Furthermore, we could demonstrate that inhibition of ERK by using a specific inhibitor effectively attenuated acute lung injury. We could show that the anti-inflammatory action of U0126 may be mediated by reduced lung neutrophilia and hyperpermeability, as well as decreased levels of pro-inflammatory cytokines and chemokines. Since effective anti-inflammatory pharmacotherapy for COPD is still not available and MAPK inhibitors are in advanced clinical trials for other chronic inflammatory diseases such as rheumatoid arthritis, ERK inhibition may represent a new treatment opportunity for COPD.

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