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Dual Effects of p38 MAPK on TNF-Dependent Bronchoconstriction and TNF-Independent Neutrophil Recruitment in Lipopolysaccharide-Induced Acute Respiratory Distress Syndrome

Silvia Schnyder-Candrian,* Valerie F. J. Quesniaux,* Franco Di Padova,‡ Isabelle Mailet,‡ Nicolas Noulin,* Isabelle Coullin,‡ René Moser,§ Francois Erard,* B. Boris Vargaftig,* Bernhard Ryffel,¶ and Bruno Schnyder**

The administration of endotoxins from Gram-negative bacteria induces manifestations reminding of acute respiratory distress syndrome. p38 MAPKs have been implicated in this pathology. In this study, we show that the specific p38 α,β MAPK inhibitor, compound 37, prevents LPS-induced bronchoconstriction and neutrophil recruitment into the lungs and bronchoalveolar space in a dose-dependent manner in C57BL/6 mice. Furthermore, TNF induction and TNF signals were blocked. In TNF-deficient mice, bronchoconstriction, but not neutrophil sequestration, in the lung was abrogated after LPS administration. Therefore, TNF inhibition does not explain all of the effects of the p38 MAPK inhibitor. The p38 α,β MAPK inhibitor also prevented LPS-induced neutrophilia in TNF-deficient mice. In conclusion, LPS provokes acute bronchoconstriction that is TNF dependent and p38 MAPK mediated, whereas the neutrophil recruitment is independent of TNF but depends on LPS/TLR4-induced signals mediated by p38 MAPK. The Journal of Immunology, 2005, 175: 262–269.

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irway exposure to endotoxins (LPS) in mice induces acute pulmonary inflammation with recruitment and activation of macrophages and neutrophils and local TNF production (1). The aerogenic exposure to the Gram-negative component LPS induces vascular leakage and bronchopulmonary hyperreactivity both spontaneously (termed bronchoconstriction) and in response to methacholine (1). These features are also typical of acute respiratory distress syndrome (ARDS) in man, a life-threatening situation caused by infection with Gram-negative bacteria (2).

The molecular relationship between the nonallergic airway inflammation (e.g., induced by LPS) and bronchopulmonary functional disturbances (e.g., bronchoconstriction) has not been investigated thoroughly. In clinical studies, mechanical ventilation increases cytokine production. A correlation between the levels of cytokines and the severity of ARDS is recognized, but the role of cytokines in acute bronchoconstriction is not clear (3). Cyclic stress of ventilation turns on the same intracellular signaling molecules as those triggered by inflammatory cytokines and LPS. They include enzymes of the NF-κB pathway and stress kinases of the MAPK family, in particular p38 (3). p38 MAPK is one of three distinct families of MAPKs (p42/44 or Erk kinase, JNK kinase, and p38 kinase) (4, 5). p38 MAPK α–δ are ubiquitous and highly conserved serine-threonine protein kinases. They regulate transcription through phosphorylation of transcription factors such as ATF-2, p53, C/EBP-homologous protein, muscle-specific transcription factor-2, and Stat-1 (6). In particular, transcription of the TNF-α gene is dependent on p38 MAPK. LPS-induced IL-1 and TNF-α production is also regulated at the translational level (7), and p38 MAPK enhances mRNA stability of the cytokines, which in some cases is mediated by MAPK-activated protein kinase-2 (MAPKAP kinase-2) (8). The p38 MAPK substrate MAPKAP kinase-2 colocalized with F-actin in the leading front of the polarized neutrophils, primarily in the lamelipodia regions (9). We showed recently that p38 MAPK inhibitors block chemotaxis of neutrophils in vitro (10, 11). Accordingly, p38 MAPK regulates neutrophil chemotaxis in part by activating MAPKAP kinase-2, which in turn phosphorylates an F-actin cross-linking protein such as lymphocyte-specific protein-1.

Systemic administration of p38 MAPK inhibitor decreased neutrophil accumulation and IL-6 release in LPS-induced pulmonary inflammation (6, 12). With the inhibitor directly affecting the neutrophil chemotaxis (10), the decreased neutrophil accumulation is likely due to a systemic effect of the inhibitor; reducing neutrophil extravasation from the circulation. In this study, the local effect of the novel, specific p38 MAPK inhibitor compound 37 was addressed. This new inhibitor (13) is highly specific for the p38 α,β MAPK, in contrast to other p38 inhibitors (14). Although most in vivo and in vitro studies demonstrated anti-inflammatory effects after p38 MAPK inhibition, a few reports have identified conditions in which p38 MAPK inhibitors enhance inflammatory responses (15, 16). The

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results suggested that various cells may use the p38 MAPK signaling cascade with different outcomes. In the current study, we analyzed the implication of p38 MAPK on several aspects of LPS-induced ARDS, such as inflammatory cell recruitment (17), cytokine release, and bronchoconstriction.

Materials and Methods

**Mice**

C57Bl/6 wild-type mice, IL-1R-deficient mice (IL-1R1 knockout (ko)) (18), TNF-deficient mice (TNF ko) (19), and TLR4-deficient mice (TLR4 ko) (20), all backcrossed on the C57Bl/6 background, were bred in our specific pathogen-free animal facility at the Centre National de la Recherche Scientifique. For experiments, adults (6- to 8-week-old) animals of 23- to 25-g body weight were kept in isolated ventilated cages. All protocols complied with the French Government’s ethical and animal experiment regulations.

**Compound 37**

The small m.w. inhibitor compound 37, a substituted imidazole(1,2-b)pyridine, was prepared as described previously (13). In brief, with the aim to develop orally and intranasally available medication, we have recently described a library comprising 18 compounds delivering four novel p38a MAPK inhibitors with enzymatic, cellular, and in vivo activities in three models of rheumatoid arthritis (13). The established novel MAPK inhibitor compound 37, used in the current study, interacts with the p38a binding pocket forming a salt bridge with Asp166 (13) and exhibits a long half-life in vivo (12.5 h). Compound 37 was dissolved in 100% DMSO at the concentration of 200 mg/ml and diluted in saline (0.9% NaCl) solution and culture medium, resulting in a 0.5%/v/v DMSO concentration for in vivo applications and a 0.003%/v/v DMSO concentration for cell stimulations, respectively. Identical DMSO concentrations were used in the vehicle solution, which was used for both LPS and compound 37. Administration of vehicle alone yielded no response in any of the parameters measured, either in vitro or in vivo (data not shown). Also, compound 37 did not affect the baseline parameters in the absence of LPS (data not shown).

**Analysis of kinase and cyclooxygenase (COX) activities in vitro**

Kinase solutions were preincubated with the inhibitor compound 37 diluted or the vehicle solution for 15 min at 37°C. The phosphorylated forms of human and mouse His-p38a MAPK (10 ng/well) were used to phosphorylate the immobilized substrate GST-ATF-2 in the presence of 120 μM ATP. Further, phosphorylated forms of human His-p38g (30 ng/well), His-p38c (3 ng/well), and His-JNK1 (30 ng/well) were used to phosphorylate the immobilized substrate GST-ATF-2 in the presence of 120 μM cold ATP. The phosphorylated GST-ATF-2 was detected by direct labeling using rabbit polyclonal antibodies. Similarly, an active form of human GST-MK6b kinase (30 ng/well) was used to phosphorylate the immobilized substrate GST-p38a in the presence of 12 μM cold ATP. The phosphorylated GST-p38a was detected by indirect labeling using rabbit polyclonal antibodies.

Four hundred units of COX-1 or 80 U of COX-2 were diluted in 10 ml of suspension buffer (pH 7.4) and kept on ice for 20 min. One hundred microliters of enzyme solution were then preincubated with 100 μl of the inhibitor compound 37 diluted for 15 min at 37°C. The reaction was started by adding 100 μl of substrate solution containing 9 μM arachidonic acid and, after 4 min, 75 μl of the reaction mixtures analyzed for PGE2 contents in ice-cold enzyme immunoassay solution according to the manufacturer’s instructions (Cayman Chemicals).

**Analysis of p38 kinase activity in vivo**

Mice were challenged with 20 μg of LPS intranasally under mild anesthesia in the presence or absence of 50 μg of p38 MAPK inhibitor compound 37. Five and 20 min after the challenge, mice were killed by CO2 inhalation. The lungs were excised and frozen at −80°C until further use.

**Experimental protocol of ARDS**

Mice were given a low dose of ketamine/xylazine (1.25 mg/ml:0.5 mg/ml) by the i.v. route in a volume of 150 μl. Holding the mice in an upright position, 40 μl of compound 37 or the corresponding vehicle solution was administered by the intranasal route (20 μl in each nostril) using an ultra-fine pipette tip. One hour later, the mice were anesthetized a second time as described above, and 40 μl of LPS (Escherichia coli, serotype O111:B4; Sigma-Aldrich) diluted in saline was administered intranasally. Identical results were obtained when compound 37 was applied simultaneously with LPS in a final volume of 40 μl. Four and 24 h after stimulation, the mice were killed and analyzed (see below). Mice were given a high dose of ketamine/xylazine i.p. and bled out. Via a tracheal cannula, the lungs were washed four times with 0.5 ml of ice-cold PBS (branched polyvinyl alcohol (BAPL) fluid). BAL fluid was analyzed for cell composition and protein and cytokine quantifications. After BAL, the lung was perfused via heart puncture with ISOTON II acid-free balanced electrolyte solution (Beckman Coulter). One-half of the lung was stored at −20°C for the myeloperoxidase (MPO) assay, and the other half was fixed in 4% buffered formaldehyde overnight for histology analysis. Results from groups of four animals are shown in the figures. Experiments were performed at least twice with each n = 4 mice per group.

**Primary bone marrow-derived macrophage cultures and stimulation**

Murine bone marrow cells were isolated from femurs. Cells were differentiated into macrophages by culturing them at 106 cells/ml for a total of 10 days in DMEM (Sigma-Aldrich) supplemented with 20% horse serum and 30% L929 cell-conditioned medium as a source of M-CSF, as described previously (21). After 7 days, cells were washed and recultured in fresh medium for another 3 days. The cell preparation contained a homogenous population of macrophages. For the experiment, the cells were suspended in medium containing 0.25% FCS, plated in 96-well microtiter plates with round bottoms (103 cells/well), and stimulated after adherence with LPS (E. coli, serotype O111:B4; at 100 ng/ml; Sigma-Aldrich). Cell supernatants were harvested after 24 h and analyzed directly for cytokine quantification or stored frozen.

**Primary pulmonary macrophages**

Mice were killed by CO2, and the lungs were perfused with 10 ml of Ca2+- and Mg2+-free PBS supplemented with 0.02% EDTA. The lungs were excised, minced, and digested for 2 h in HBSS containing 200 U/ml Collagenase II (Invitrogen Life Technologies), 50 U/ml DNase, 100 U/ml penicillin, and 100 μg/ml streptomycin. The homogenate was passed through 100-μm pore size filters, and the cell suspension was washed three times with PBS supplemented with 2% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin and centrifuged at 200 × g. The pellet was reconstituted, and macrophages were removed by adherence for 90–120 min at 37°C. The macrophages were detached with cold PBS and cultivated at the density of 2 × 104 cells/well in 96-well plates in RPMI 1640 containing 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.5% heat-inactivated FCS (PAA Laboratories). Stimulations were performed with 3 ng/ml murine TNF (R&D Duoset) and 100 ng/ml LPS (E. coli, serotype O111: B4; Sigma-Aldrich) in the presence or absence of the p38 inhibitor.

**Airways resistance**

The airways resistance was evaluated by whole-body plethysmography (22). Bronchoconstriction was investigated over a period of 3–6 h after LPS application (10 μg of LPS intranasally per mouse). Unrestrained conscious mice were placed in whole-body plethysmography chambers (Buxco Electronic). Mean airway bronchoconstriction was estimated by the Enhanced Respiratory Pause (Penh) index. Penh can be conceptualized as the phase shift of the thoracic flow and the nasal flow curves; increased phase shift correlates with increased respiratory system resistance.

Penh is calculated by the formula Penh = (Te/Tr − 1) × PEF/PIF, where Te is expiratory time, RT is relaxation time, PEF is peak expiratory flow, and PIF is peak inspiratory flow.
Table I. Inhibition of p38 MAPK and profiling of other MAP kinases and related enzymesa

<table>
<thead>
<tr>
<th>Enzyme Tested</th>
<th>IC50 Value (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>p38α MAPK (human)</td>
<td>0.018 ± 0.002</td>
</tr>
<tr>
<td>p38α MAPK (mouse)</td>
<td>0.006 ± 0.002</td>
</tr>
<tr>
<td>p38β2 MAPK</td>
<td>0.030 ± 0.009</td>
</tr>
<tr>
<td>p38δ MAPK</td>
<td>10.137 ± 1.321</td>
</tr>
<tr>
<td>INK1 MAPK</td>
<td>1.880 ± 0.579</td>
</tr>
<tr>
<td>MKK6b (kinase of p38)</td>
<td>&gt;10</td>
</tr>
<tr>
<td>COX-1</td>
<td>&gt;10</td>
</tr>
<tr>
<td>COX-2</td>
<td>&gt;10</td>
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aUsing in vitro phosphorylation assays, the IC50 values of p38 inhibitor compound 37 for the different p38 MAP kinases and the related enzymes were determined. The IC50 values were calculated based on three independent experiments and are given as means ± SEM.

Bronchoalveolar lavage

BAL fluids were prepared by washing the lungs four times with 0.5 ml of ice-cold PBS. The cells were sedimented by centrifugation at 400 × g for 10 min at 4°C. The supernatant of the first lavage (cell-free BAL fluid) was stored at −70°C for cytokine analysis. The cell pellets were resuspended and pooled, and an aliquot was stained with Turk’s solution and counted. All cell preparations were analyzed morphologically after centrifugation on microscopic slides. Air-dried preparations were fixed and stained with H&E. The sections were examined with a Leica microscope (×20 and ×40 magnification). The peribronchial infiltrate and the smooth muscle hyperplasia were assessed by a semiquantitative score (0 –3) by two observers.

Lung histology

The organs were fixed in 4% buffered formaldehyde overnight and embedded (23) in paraffin. Three-micrometer lung sections were stained with H&E. The sections were examined with a Leica microscope (×20 and ×40 magnification). The peribronchial infiltrate and the smooth muscle hyperplasia were assessed by a semiquantitative score (0–3) by two observers.

Measurement of cytokines by ELISA

TNF, IL-6, IL-10, and KC (CXCL1) contents in cell culture supernatants and the BAL fluid were evaluated by ELISA according to the manufacturer’s instructions (R&D Dueset).

Vascular leakage

The vascular leakage was assessed by measurements of albumin in the bronchoalveolar fluid 90 min after intranasal challenge by ELISA according to the manufacturer’s instructions (SPBio).

MPO assay

MPO activity in the lung tissue was evaluated as described by Lefort et al. (22). In brief, the frozen lung was homogenized for 30 s in 1 ml of ice-cold PBS with a polytron mixer. The extract was centrifuged (10,000 × g, 10 min at 4°C), and the supernatant was discarded. The pellet was resuspended in 1 ml of PBS-HTAB-EDTA (PBS containing 0.5% hexadecyltrimethyl ammonium bromide (HTAB; Sigma-Aldrich) and 1 mM EDTA), homogenized for 30 s, and centrifuged. One hundred microliters of the supernatant were placed in a tube, together with 2 ml of HBSS (with Ca2+ and Mg2+; Invitrogen Life Technologies), 200 μl of PBS-HTAB-EDTA, 100 μl of o-dianisidine (1.25 mg/ml H2O; Sigma-Aldrich), and 100 μl of H2O2. After a 15-min incubation at 37°C under agitation, the reaction was stopped by transfer on ice and the addition of 100 μl of NaOCl (1%; Sigma-Aldrich). The MPO activity was quantified as absorbance at 460 nm.

Statistical analysis

Data are presented as means and SD, with the exception of the data of enzyme activities, which are presented as means and SEM. Statistical significance was determined by the Student’s t test for comparisons of two groups. Multigroup comparisons were performed using one-way ANOVA multiple comparisons among means, with the Tukey-Kramer multiple comparisons tests. p values of <0.05 were considered statistically significant.

Results

Compound 37 inhibits p38 α,β MAPK

The use of specific p38 MAPK inhibitors is instrumental in evaluating the role of the p38 MAPK in vivo, in particular because no p38 gene-deficient mice are available (24). Novel p38 MAPK inhibitors acting primarily on the p38 α,β MAPK with IC50 values in the nanomolar range have been described recently (13). The small m.w. imidazol(1,2-b)pyridine (compound 37) inhibits the p38 α,β MAPK with IC50 values between 6 ± 2 and 30 ± 9 nM (Table I). Additional kinases including additional MAPK members were not or only marginally inhibited by compound 37 with IC50 values between 1.88 and >10 μM (Table I) (13). Compound 37 did not inhibit the activating kinase of p38 MAPK, MKK6b, indicating that it impaired the activity but not the activation of p38.
MAPK (Table I). Similarly, previously described p38 MAPK inhibitors (e.g., pyridinylimidazole SB203580) also inhibited the activity but not the activation of p38 MAPK (25). Based on these characteristics, the in vivo effect of the inhibitor was analyzed assessing the phosphorylation of the substrate of the p38 kinase MAPKAP kinase-2, rather than the phosphorylation of p38 itself. Intranasal application of 20 μg of LPS induced phosphorylation of MAPKAP kinase-2 at threonine residue 334 within 5 min and, to a lesser extent, after 20 min (Fig. 1). Application of LPS simultaneously with 50 μg of the p38 inhibitor prevented the MAPKAP kinase-2 phosphorylation. We published recently (14) that in contrast to the previously described SB203580, compound 37 did not inhibit human cytochrome P450 isoenzymes. A predecessor of SB203580 (14) also inhibited COX-1 activity, which represented an unacceptable ulceration risk and prompted the design and synthesis of selective p38 inhibitors devoid of COX-1 inhibition. In fact, compound 37 did not inhibit COX-1 or COX-2 up to 100 μM (Table I). Indomethacin, used as a control, had an IC\textsubscript{50} value on COX-1 of 0.017 ± 0.001 μM and on COX-2 of 0.251 ± 0.014 μM. Therefore, compound 37 used in this study showed a selectivity toward p38 α,β MAPK and inhibited phosphorylation and activation of the p38 substrate MAPKAP kinase-2 in vivo.

**Inhibition of LPS-induced TNF synthesis by the MAPK inhibitor compound 37 in primary macrophages**

The efficacy of the p38 MAPK inhibitor compound 37 on murine macrophage cytokine synthesis was first assessed using primary bone marrow-derived macrophages in vitro. Macrophages were stimulated with 100 ng/ml LPS for 24 h in the presence or absence of the inhibitor. Compound 37 reduced TNF synthesis in a dose-dependent manner but failed to reduce LPS-induced IL-6 secretion (Fig. 2, A and B). Compound 37 inhibited the synthesis of IL-10 and chemokine KC (CXCL1), a neutrophil chemoattractant (Fig. 2, C and D). The cell viability was not altered at the p38 inhibitor concentrations used, as assessed by mitochondrial activity (MTT assay); the specific values of OD\textsubscript{630} 0.55–0.60, were not statistically different in the presence and absence of compound 37.

**LPS-induced TNF and IL-6 secretion as well as protein leakage in the lungs is dependent on p38 MAPK signaling**

Because the p38 MAPK signaling is critical for LPS-induced proinflammatory cytokine synthesis in bone-marrow macrophage cultures, we delineated its role in lung-derived macrophages and studied whether in vivo administration of compound 37 reduces TNF levels in the bronchoalveolar fluid after stimulation with LPS. The intranasal administration of compound 37 1 h before intranasal application of LPS (10 μg per mouse) significantly reduced TNF levels in the BAL fluid (Fig. 3A) in a concentration-dependent manner. This effect was already noted after 90 min (up to 50% reduction) and was more pronounced at 4 h (Fig. 3A). The local administration of LPS induced no alterations of systemic TNF levels, unlike systemic LPS administration, which increases TNF serum levels (13, 26). Compound 37 also reduced IL-6 production in the lung significantly at 90 min (up to 40% reduction) and at 4 h after stimulation (Fig. 3B).

Formation of protein-rich alveolar edema after damage to the integrity of the alveolar-capillary barrier of the lung in human....

**FIGURE 3.** p38 MAPK mediates LPS-induced bronchial TNF and IL-6 secretion. The p38 inhibitor compound 37 reduces TNF and IL-6 secretion into BAL fluid. Mice were intranasally treated with vehicle alone or with the indicated doses of p38 inhibitor compound 37 1 h before intranasal application of 10 μg of LPS. Four hours after LPS application, the BALs were performed. TNF (A) and IL-6 (B) release into the alveolar space was determined by ELISA. Vascular leakage was analyzed 90 min after LPS administration by measuring the albumin concentration in the BAL (C). The results represent n = 8 mice per group, pooled from two independent experiments. *p < 0.05.

**FIGURE 4.** p38 MAPK mediates LPS-induced TNF and IL-6 secretion by lung macrophages. Pulmonary macrophages were harvested from either wild-type (A) or TNF-deficient (B) mice and stimulated for 24 h with either 3 ng/ml murine TNF or 100 ng/ml LPS, in the presence or absence of 0.1 μg/ml compound 37. TNF and IL-6 concentrations in the supernatant were determined by ELISA and are expressed in percentage of the full response in the absence of inhibitor (100% accounting for 310 and 425 pg/ml for TNF and IL-6 production, respectively). Vehicle or compound 37 alone yielded no cytokine production. The data show mean values ± SD of triplicates and are representative of two independent experiments. *p < 0.05.
ARDS impairs alveolar ventilation and in turn decreases lung compliance (27). We therefore analyzed the leakage of circulating albumin into the alveolar space, assessed in the BAL fluid. Administration of 10 μg of LPS intranasally to C57BL/6 mice induced an increase in BAL albumin content after 90 min (Fig. 3C). The presence of compound 37 prevented the LPS-induced protein leakage at doses of 0.5 and 5 μg per mouse (*p < 0.01) (Fig. 3C), indicating that LPS-induced vascular leakage is signaled through p38 MAPK postreceptor events.

In a next set of experiments, lung macrophages were harvested from naive mice, and the effect of compound 37 on induction of TNF and the TNF-induced IL-6 production was studied. LPS-induced TNF production was inhibited by 83 ± 6%, and IL-6 synthesis was inhibited by 63 ± 3% at 0.1 μg/ml compound 37 (Fig. 4A). Also in TNF-deficient mice, the LPS-induced IL-6 secretion was inhibited by 73 ± 11% in the presence of compound 37 (Fig. 4B). Furthermore, the effect of the p38 inhibitor on TNF-induced IL-6 secretion was analyzed. Compound 37 inhibited TNF-induced IL-6 secretion by 66 ± 4%. Therefore, pulmonary macrophages

**FIGURE 5.** LPS-induced bronchoconstriction is p38 MAPK and TNF dependent. The p38 MAPK inhibitor compound 37 completely blocks LPS-induced bronchoconstriction. A, Wild-type mice were treated intranasally with the p38 inhibitor and challenged with 10 μg of LPS. B, Groups of wild-type mice, TNF-deficient mice (TNF ko), and IL-1R1-deficient mice (IL-1R1 ko) were treated intranasally with 10 μg of LPS. The saline control of wild-type mice is shown, which is also representative for the saline controls of TNF- and IL-1R1-deficient mice as well as for the compound 37 added alone. The bronchoconstriction was recorded for 3 h using whole-body plethysmography. Penh values are given as the mean ± SD of n = 8 mice per group pooled from two independent experiments.

**FIGURE 6.** LPS-induced lung neutrophilia is mediated by p38 MAPK. The p38 MAPK inhibitor compound 37 blocks LPS-induced neutrophil recruitment in the bronchial space and in the lung. Animals were treated with the indicated concentrations of the p38 inhibitor and 10 μg of LPS (as in Fig. 3) and killed after 4 h. Total neutrophil counts in the BAL fluid (BALF) are given in A, and neutrophil MPO activity (indicated in OD460) in the respective lung tissue is shown in B. Results are the mean ± SD of n = 8 mice per group, pooled from two independent experiments. *p < 0.05.

**FIGURE 7.** Pulmonary neutrophil infiltration after LPS treatment is p38 MAPK dependent. The p38 MAPK inhibitor compound 37 prevents the recruitment of neutrophils in the lung, as assessed by histological analysis. Mice were treated with NaCl, 10 μg of LPS alone, and LPS combined with 5 or 50 μg of p38 inhibitor (inh.), as in Fig. 6. Representative H&E staining of lung sections are shown. Magnification, ×20.
are more susceptible to compound 37 compared with bone marrow-derived macrophages. In addition, compound 37 inhibited not only the production of TNF but also TNF-induced signaling leading to IL-6 secretion.

LPS-induced bronchoconstriction is p38 MAPK and TNF dependent

Administration of 10 μg of LPS intranasally induced an acute bronchoconstriction increasing Penh values within 90–120 min, which lasted for 3–4 h. LPS signaled through TLR4 receptors, because mice deficient in TLR4 did not respond; Penh values were not increased in LPS-treated, TLR4-deficient mice and the NaCl-treated groups. LPS signaled through p38 MAPK postreceptor events, because administration of compound 37 to C57BL/6 mice completely prevented the LPS-induced bronchoconstriction at doses of 50 and 5 μg per mouse, whereas the 0.5-μg dose was slightly less effective (p < 0.01) (Fig. 5A). Compound 37 did not affect bronchoconstriction to methacholine as assessed in naive C57BL/6 mice nebulized with 60 mM methacholine. Methacholine-induced Penh values (of maximally 2 arbitrary units) were similar in the presence and absence of compound 37. Similarly, the p38 inhibitor had no effect on bronchohyperreactivity to methacholine in OVA-immunized and -challenged BALB/c mice (S. Schnyder-Candrian, I. Maillet, B. Ryffel, and B. Schnyder, unpublished data) (28). The fact that compound 37 affects LPS-induced, but not methacholine-induced, bronchoconstriction supports the concept that both functions are mediated by distinct mechanisms and that only LPS-induced bronchoconstriction is p38 MAPK dependent.

To address the question whether a correlation exists between TNF induction and bronchoconstriction, both being suppressed by compound 37, TNF-deficient mice were studied in the model of LPS-induced bronchoconstriction. In the absence of TNF, LPS was unable to cause bronchoconstriction (Fig. 5B), suggesting that TNF mediates LPS-induced bronchoconstriction. In the absence of the IL-1R, as assessed in IL-1R1-deficient mice (IL-1R1 ko), LPS-induced bronchoconstriction was intact (Fig. 5B), implicating that LPS-induced bronchoconstriction is TNF but not IL-1 dependent.

LPS-induced neutrophilia is p38 MAPK dependent but independent of TNF

Intranasal administration of LPS induced a significant increase of the total cell number in the lung, mainly neutrophils, already at 4 h. This neutrophilia was TLR4 receptor dependent, as assessed in TLR4-deficient mice, with 5 × 10^6 cells/BAL in TLR4-deficient mice vs 9 × 10^5 cells/BAL in C57BL/6 wild-type mice (p < 0.01). Compound 37 inhibited the neutrophil recruitment into the BAL in a dose-dependent manner (Fig. 6A). It decreased neutrophil MPO activity in the lung tissue (p < 0.01) (Fig. 6B). Neutrophil infiltration into the lung tissue was also clearly inhibited by compound 37, as assessed in histological sections (Fig. 7).

To address the question whether a correlation exists between TNF induction and neutrophilia, both being suppressed by compound 37, TNF-deficient mice were studied. However, both wild-type and TNF-deficient mice recruited similar numbers of total cells in the bronchoalveolar fluid (Fig. 8A). The increased infiltration consisted mainly in neutrophils (Fig. 8B) and correlated with the MPO activity found in the lung tissue (C). Compound 37 significantly inhibited the total cell number and the neutrophil counts in the bronchoalveolar fluid of LPS-challenged wild-type or TNF-deficient mice (p < 0.01) (Fig. 8, A and B). The reduced neutrophil counts correlated with reduced MPO activity in the lung tissues (Fig. 8C). To investigate the molecular target mechanism of how compound 37 inhibits neutrophil recruitment, secretion of the major neutrophil chemoattractant KC (CXCL1) was measured. Both wild-type and TNF-deficient mice secreted similar amounts of KC in the bronchoalveolar fluid after intranasal LPS stimulation (Fig. 8). Compound 37 significantly inhibited the KC secretion in the bronchoalveolar fluid of LPS-challenged wild-type or TNF-deficient mice (p < 0.01) (Fig. 8).

Thus, the neutrophil recruitment to the lung is TNF independent, but it is dependent on p38 MAPK signaling, in line with the secretion of the neutrophil chemoattractant KC (CXCL1).

Discussion

p38 MAPK is a critical enzyme for cytokine TNF production and is currently targeted for anti-inflammatory therapy. However, contradictory and insufficient data are available in vivo. We studied the implication of p38 α,β MAPK in different aspects of LPS-induced ARDS using a new specific p38 α,β inhibitor, compound 37. LPS-induced ARDS manifestations like bronchoconstriction, local TNF production, and neutrophil chemoattractant KC...
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