Chapter 6

Intrapulmonary administration of a p38 mitogen activated protein kinase inhibitor partially prevents pulmonary inflammation

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Submitted
Abstract

Background:
Gram-positive and gram-negative bacteria are common causative agents of respiratory tract infection. Lipopolysaccharide (LPS) is a component of the gram-negative cell wall and a strong inducer of inflammation. The main proinflammatory component of the gram-positive bacterial cell wall is lipoteichoic acid (LTA). The protein kinase p38 mitogen activated protein kinase (MAPK) plays an important role in the inflammatory process induced by these two bacterial structures.

Aim:
We here sought to establish the impact of local p38 MAPK inhibition on lung inflammatory responses induced by LPS and LTA. We investigated the effects of direct intrapulmonary delivery of a p38 MAPK inhibitor on local LPS and LTA induced airway inflammation in mice.

Results:
In vitro, BIRB 796 reduced LPS induced p38 MAPK phosphorylation in alveolar macrophage and respiratory epithelial cell lines and diminished cytokine/chemokine release. In vivo, BIRB 796 circumvented p38 MAPK phosphorylation in both LPS and LTA induced inflammation. Cellular influx was not affected. Lung TNF-α, IL-6, MIP-2 and LIX production was reduced in LPS induced inflammation but not in lung inflammation by LTA. BIRB 796 reduced total protein and IgM in bronchoalveolar lavage fluid after LTA instillation, while enhancing TATc and D-dimers in LPS- and LTA induced inflammation.

Conclusion:
These results taken together with earlier studies on systemic administration of p38 MAPK inhibitors in rodents and humans suggest that direct intrapulmonary delivery of a p38 MAPK inhibitor is less effective in inhibiting inflammation and is associated with unexpected procoagulant effects in the bronchoalveolar space.
Introduction

Bacterial pneumonia is a major cause of morbidity and mortality [1]. Both gram-positive and gram-negative bacteria are common causative agents in respiratory tract infection [1,2,3]. Lipopolysaccharide (LPS) is a component of the gram-negative cell wall and a strong inducer of inflammation [4]. The most important proinflammatory component of the gram-positive bacterial cell wall is lipoteichoic acid (LTA) [5]. The local effects of LPS and LTA within the lung have been implicated to contribute to the acute lung inflammation that accompanies pneumonia [6]. The inflammatory process is pivotal in host-defense against invading pathogens. However, hyper-inflammation is unwanted, as it may be detrimental the host [7]. The protein kinase p38 mitogen activated protein kinase (MAPK) is involved in the regulation of inflammatory mediator synthesis [8]. Four isoforms of MAPK are known that share >60% sequence homology [9]. The α and β isoforms are ubiquitously expressed [10]. P38-γ is mostly expressed in skeletal muscle; p38-γ is predominantly expressed in lungs, kidneys, testis, pancreas, and small intestine [11]. Both LPS and LTA can trigger p38 MAPK signaling [4,12]. LPS activates cells through stimulation of the Toll-like receptor (TLR) 4 – MD2 receptor complex, whereas LTA signals through TLR 2 [13].

We previously observed p38 MAPK signaling activity, using kinase arrays, in lungs of mice with pneumonia caused by Klebsiella (K.) pneumoniae, a common gram-negative respiratory pathogen [14]. P38 MAPK activity influences mainly the expression of pro-inflammatory mediators (e.g. Tumor Necrosis Factor (TNF) α/Interleukin (IL)-6/IL-8/Cytochrome c oxidase subunit II (COX2)) [15] and thus is a potential target to prevent hyper-inflammation. Previous studies examined systemic p38 MAPK inhibition in lung inflammation models and found beneficial anti-inflammatory effects (e.g. reduced PMN recruitment) [16,17,18,19].

The systemic administration of p38 MAPK inhibitors may cause undesired effects at body sites distant from local inflammatory processes. Therefore, we here sought to establish the impact of local p38 MAPK inhibition on lung inflammatory responses induced by LPS and LTA. In particular, we determined if direct intrapulmonary delivery of a p38 MAPK inhibitor would exert local anti-inflammatory effects after administration of LPS and LTA via the airways. We used BIRB 796, an inhibitor of all isoforms of p38 MAPK [20], of which our laboratory previously demonstrated its capacity to potently inhibit systemic inflammatory responses elicited by intravenous LPS in healthy humans [21].

Material and methods

In vitro studies

Polymorphnuclear cells (PMNs) were isolated from EDTA anticoagulated blood from healthy subjects using Polymorphprep (Axis-Shield, Oslo, Norway). 2x10⁵ PMNs were seeded on 24 wells plates (Greiner Bio-One, Monroe, NC) and treated with 10 µM BIRB 796 (Axon medchem, Groningen, The Netherlands) or vehicle (0.01% DMSO) in presence of 100 ng/ml LPS (from E. coli. 0111:B4 ; Invivogen, San Diego, CA). After 0, 15, 30 and 120 minutes cells were lysed with lysis buffer (Cell Signaling Technology, Danvers, MA) and 3x Laemmli buffer
was added prior to incubating the samples at 95 °C for 5 minutes. A similar approach was used to obtain samples to measure the phosphorylation levels of p38 MAPK in MH-S (mouse alveolar macrophage cell line; American Type Culture Collection, Rockville, MD), MLE-12 and MLE-15 cells (mouse lung epithelial cell lines; kindly provided by Jeffrey Whitsett, Division of Pulmonary Biology, Department of Pediatrics, Cincinnati Children’s Hospital Medical Center and the University of Cincinnati College of Medicine, Cincinnati, Ohio). 1x10⁵ cells were seeded in a 24 well plate (Greiner Bio-One). After 24 hours, MH-S and MLE-15 cells were gently washed and stimulated with 100 ng/ml LPS (purified from *E. coli*. 0111:B4; Invivogen, San Diego, CA). Since MLE-12 cells merely react upon gram-positive stimulation (data not shown), these cells were stimulated with 10 μg/ml *Staphylococcus (S.) aureus* LTA (endotoxin level: <1.25 EU/mg; Invivogen). Simultaneously, cells were treated with 10 μM BIRB 796 or vehicle (0.01% DMSO). In these experiments, MH-S cells were lysed 0, 15, 30, 60 and 120 minutes after stimulation with lysis buffer (Cell Signaling Technology) and 3x Laemmlli buffer was added prior to incubating the samples at 95 °C for 5 minutes. In MLE-12 and MLE-15 cell-lines an additional 240 minute time point was included.

To test the effect of BIRB 796 on cytokine responses, 1x10⁵ MH-S, MLE-12 and MLE-15 cells were seeded in a 24 well plate (Greiner Bio-One) and after 24 hours stimulated as indicated above. After 4 and 8 hours of stimulation, supernatants were harvested for ELISA. Cell viability was assessed by adding MTT ([3-(4,5-methylthiazol-2-yl)-2,5-dipheyl-tetrazolium bromide]; Sigma-Aldrich) reagent to all wells for 60 min. Supernatants were discarded and the cells were lysed in acidic isopropanol (Merck, Darmstadt, Germany). Absorbance was measured at 570 nm.

**Mouse model of lung inflammation**

Female C57BL/6 mice aged 10 to 11 weeks were purchased form Charles River (Maastricht, The Netherlands). The Animal Care and Use Committee of the University of Amsterdam approved all experiments. For intrapulmonary delivery, mice were anesthetized by isoflurane (Upjohn Ede, The Netherlands) and 10 mg/kg BIRB 796, was instilled intranasally (i.n.) in 50 μl 10% DMSO saline solution; control mice received 50 μl 10% DMSO without BIRB 796. In order to induce lung inflammation, mice were anesthetized with isoflurane (Upjohn) and 1 μg LPS (purified from *K. pneumoniae*; Sigma, St. Louis, MO) or 100 μg LTA (Invivogen), diluted in 50 μl of sterile saline was instilled i.n.. At different time points (LPS: 3; 6; 16 hours, LTA: 6 hours) after pulmonary challenge, mice were anesthetized with Domitor (Pfizer Animal Health Care, Capelle aan der IJssel, The Netherlands; active ingredient medetomidine) and Nimatek (Eurovet Animal Health, Bladel, The Netherlands; active ingredient ketamine) and sacrificed by cardiac puncture followed by cervical dislocation. Sham samples were obtained from naïve mice.

**Bronchoalveolar lavage**

To obtain bronchoalveolar lavage (BAL) fluid the trachea and lungs were exposed through a midline incision and the right lung was disconnected from the main airways. The trachea was cannulated with a 22G Abbocath-T catheter (Abbott, Sligo, Ireland); the left lung was instilled with two times 0.4 ml sterile PBS and the fluid was retrieved and weighed. Total cell counts were determined with a Coulter cell counter (Beckman Coulter, Fullerton, CA);
differential cell counts were performed on Giemsa stained cytospin preparations. For western blot, cells not used for cytospins were taken up in Laemmli buffer, boiled at 95 °C for 5 minutes and stored at -20 °C until further use.

**Assays**

IL-6, TNF-α, keratinocyte chemoattractant (KC), LPS-induced CXC Chemokine (LIX) and macrophage inflammatory protein (MIP)-2 (R&D Systems, Minneapolis, MN) and Thrombin anti-thrombin complexes (TATc; Kordia, Leiden, The Netherlands) were measured using ELISAs according to the manufacturer’s instructions. Detection limits were: TATc: 8.8 ng/ml, 10 pg/ml for TNF-α, 31.25 pg/ml for IL-6, 15.6 pg/ml for LIX and 8.2 pg/ml for MIP-2 and KC. Total protein concentrations were measured using a DC protein assay (Bio-Rad Laboratories, Veenendaal, The Netherlands). For Immunoglobulin M (IgM) measurements 96 wells plates (Greiner Bio-One) were plated with anti-mouse Ig (SouthernBioTech, Birmingham, Alabama) in sodium carbonate buffer at 4°C overnight. Plates were blocked in 5% bovine serum albumin (Roche Applied Science, Indianapolis, IN) in phosphate buffered saline (PBS) (Fresenius Kabi, Bad Homburg, Germany) for 1 hour at room temperature and incubated with diluted samples in 1% BSA and 0.05% Tween 20 (Sigma-Aldrich) in PBS for 2 hours in 37°C. Anti-mouse IgM-HRP (SouthernBioTech) was used for detection, plates were developed with 3,3′,5,5′-tetramethylbenzidine (TMB; Invitrogen, Paisley, UK) and 0.003% H₂O₂ (Merck) and absorbance was measured at 450 nm.

**Western blotting**

Samples were loaded onto SDS-PAGE gels. After electrophoresis the content of the gels was transferred onto Immobilon-PVDF membranes (Millipore, Billerica, MA). The membranes were blocked in 5% BSA (Roche, Basel, Switzerland) in TBS-T at room temperature for 60 minutes. Phosphorylated p38 MAPK (Cell signaling Technology, Boston, MA) and Fibrinogen (Kordia, Leiden, the Netherlands) antibodies were diluted to 1:1000, β-actin (Santacruz Biotechnology, Santa Cruz, CA) was diluted 1:2000. The membranes were incubated overnight at 4°C. Next, the membranes were incubated at room temperature for 60 minutes with anti-rabbit-HRP conjugated (Cell signaling Technology) or anti-goat-HRP conjugated secondary antibody (DakoCytomation, Glostrup, Denmark). Blots were imaged using LumiLight Plus ECL (Roche, Basel, Switzerland) on a LAS 4000 chemiluminescence imager (GE Healthcare Biosciences, Pittsburgh, PA). Image quantification was performed using ImageJ software (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, http://rsb.info.nih.gov/ij/, 1997-2011).

**Statistical analysis**

Data are expressed as mean ± SEM. For *in vivo* data, two sample comparisons were performed with Mann Whitney U tests using GraphPad Prism version 5.01 (GraphPad Software, San Diego, CA). Comparisons between multiple groups were done using Kruskal-Wallis tests; if overall significant individual groups were assessed by Mann Whitney-U tests. For *in vitro* analysis, student t-tests were applied. Multiple group analysis was performed by 2-way ANOVA. P < 0.05 was considered to be statistically significant.
Results

BIRB 796 inhibits p38 MAPK phosphorylation and inflammatory mediator production in vitro

It is known that BIRB 796 inhibits both p38 MAPK activity and p38 MAPK phosphorylation [21,22]. We investigated the effect of BIRB 796 on the phosphorylation status of p38 MAPK in freshly isolated human PMNs and in three typical murine lung cell lines. In addition, we
assessed the effects of BIRB 796 on inflammatory mediator production by these cell types. The phosphorylated p38 MAPK was measured at several time points within the first minutes of exposure to LPS (as indicated in figure 1), while TNF-α or KC levels were measured after 4 and 7 hours of stimulation.

Phosphorylation dynamics of p38 MAPK in response to stimulation differed between cell types (figure 1A-D), as determined by western blotting. In PMNs and MH-S (alveolar macrophage) cells the increase in p38 MAPK phosphorylation was more rapidly normalized (<120 minutes) than in MLE-15 (respiratory epithelial) cells (<240 minutes). Notably, PMNs harvested directly after exposure to LPS (t = 0) already showed detectable p38 MAPK phosphorylation; vehicle treated MLE-12 cells displayed a sustained highly phosphorylated p38 MAPK state which further increased in time. Regardless of the cell type dependent dynamics, BIRB 796 reduced the phosphorylation levels of p38 MAPK in all cell types compared to vehicle treated cells.

Apart from p38 MAPK phosphorylation, BIRB 796 reduced TNF-α levels in MH-S (figure 1E) cell supernatants at 4 and 7 hours of LPS stimulation (P<0.001). In lung epithelial cell-lines a less pronounced effect was observed. A modest reduction in KC production by MLE-12 cells

Figure 2: Intrapulmonary BIRB 796 delivery circumvents LPS and LTA induced p38 MAPK phosphorylation

Mice were inoculated with LPS (1 μg) or LTA (100 μg) and simultaneously treated with either BIRB 796 (10 mg/kg) or vehicle. After 3, 6 and 18 hours of LPS and 6 hours of LTA inflammation animals were sacrificed; sham samples were obtained from naïve mice. Cellular content obtained from BAL fluid was lysed and p38 MAPK phosphorylation was determined by western blot. (A, B) Western blot for p-p38 MAPK of BAL cells after LPS administration. (C, D) p38 MAPK phosphorylation levels in BAL cells after LTA administration. Data are expressed as means ± SEM (n = 4-8) * = P < 0.05
Intrapulmonary p38 inhibition partially prevents inflammation (figure 1F) in BIRB 796 treated cells occurred compared to vehicle controls (P<0.01), while in MLE-15 cells BIRB 796 resulted in a trend towards a reduced KC production (figure 1G; P=0.066). Viability of these cell-lines was not statistically significantly altered due to BIRB 796 treatment (figure 2E-F inserts).

**BIRB 796 circumvents LPS and LTA induced p38 MAPK phosphorylation in vivo**

Given the strong effect observed in vitro, we applied BIRB 796 locally in murine pulmonary inflammation. BIRB 796 (10 mg/kg) was inoculated i.n. at t=0 hours combined with either *K. pneumoniae* LPS (1 μg) or *S. aureus* LTA (100 μg). At t=3, 6 and 18 hours of LPS and t=6 hours of LTA induced lung inflammation mice were sacrificed and BAL performed. From the cellular content of these lavages p38 MAPK phosphorylation states were determined by western blot. Phosphorylation of p38 MAPK increased in the vehicle group compared to sham upon both LPS and LTA stimulation (figure 2). After 18 hours of LPS inflammation phosphorylation of p38 MAPK returned to baseline. Local administration of BIRB 796 reduced p38 MAPK phosphorylation to baseline levels in response to both LPS (at t=3 and 6 hours; P<0.05) and LTA (t=6 hours, P<0.05) instillation.

**P38 MAPK inhibition does not affect intrapulmonary cellular composition during lung inflammation**

Cellular differential counts were performed on cells derived from BAL fluid (Table 1). During the course of LPS-induced inflammation the numbers of lymphocytes and PMNs increased. Lymphocyte counts were statistically significantly reduced (P<0.05) at 3 hours due to BIRB 796 treatment, while no statistically significant changes occurred in the numbers of other

<table>
<thead>
<tr>
<th>Time</th>
<th>Group</th>
<th>PMNs</th>
<th>Macrophages</th>
<th>Lymphocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 hours</td>
<td>Sham</td>
<td>0.13 ± 0.04</td>
<td>6.48 ± 0.65</td>
<td>0.30 ± 0.04</td>
</tr>
<tr>
<td>3 hours LPS</td>
<td>Vehicle</td>
<td>17.26 ± 0.59</td>
<td>3.07 ± 0.58</td>
<td>0.52 ± 0.15</td>
</tr>
<tr>
<td>6 hours LPS</td>
<td>BIRB</td>
<td>14.26 ± 1.29</td>
<td>2.3 ± 0.39</td>
<td>0.21 ± 0.02*</td>
</tr>
<tr>
<td>18 hours LPS</td>
<td>Vehicle</td>
<td>23.01 ± 1.62</td>
<td>3.01 ± 0.34</td>
<td>0.96 ± 0.17</td>
</tr>
<tr>
<td>18 hours LPS</td>
<td>BIRB</td>
<td>23.54 ± 1.57</td>
<td>3.68 ± 0.62</td>
<td>0.66 ± 0.12</td>
</tr>
<tr>
<td>6 hours LTA</td>
<td>Vehicle</td>
<td>33.06 ± 3.30</td>
<td>4.10 ± 0.54</td>
<td>0.68 ± 0.15</td>
</tr>
<tr>
<td>6 hours LTA</td>
<td>BIRB</td>
<td>37.88 ± 4.67</td>
<td>3.21 ± 0.40</td>
<td>0.84 ± 0.15</td>
</tr>
</tbody>
</table>

Mice were inoculated with LPS (1 μg) or LTA (100 μg) and simultaneously treated with either BIRB 796 (10 mg/kg) or vehicle. After 3, 6 and 18 hours of LPS and 6 hours of LTA inflammation animals were sacrificed; sham samples were obtained from naïve mice. In cytoplasm preparations of BAL PMN, macrophage and lymphocyte counts (shown in 10⁴) were determined. Data are expressed as mean ± SEM, N=4-8. * = P < 0.05 vs. vehicle.
Intrapulmonary p38 inhibition partially prevents inflammation

cell-types or time points. After 6 hours of LTA-induced inflammation, BIRB 796 intervention did not influence the cellular composition of BAL fluid in LTA-induced inflammation.

**P38 MAPK inhibition reduces TNF-α levels in LPS- but not in LTA-induced inflammation**

To determine if reduced phosphorylation state of p38 MAPK affected inflammatory mediator levels, we measured TNF-α, IL-6, KC, MIP-2 and LIX in BAL fluid (Table 2).

During the course of LPS-induced inflammation TNF-α, IL-6, KC and MIP-2 increased considerably in the untreated group. The peak values of these inflammatory mediators were observed at 3 hours. Local BIRB 796 treatment significantly reduced TNF-α levels during the entire time course of LPS-induced inflammation. IL-6 was reduced after 3 hours (P<0.5), while MIP-2 levels were diminished at 3 and 6 hours (P<0.05). Interestingly, KC levels were not affected by BIRB 796 treatment, while BIRB 796 intervention reduced the concentrations of LIX at 6 (P<0.05) and 18 hours (P<0.01).

LTA-induced inflammation resulted in only a subtle rise in inflammatory mediator levels. Apart from an increase in LIX levels (P<0.01) no statistically significant differences were observed.
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To assess lung barrier integrity, total protein and IgM levels were measured in BAL fluid (figure 3). Total protein levels in BAL fluid were reduced after 3 hours (figure 3A, P<0.05) of LPS-induced inflammation due to BIRB 796 treatment. This difference did not last, since at the 18 hour time point total protein levels were enhanced in both treatment and vehicle group. Six hours after instillation LTA, BIRB 796 reduced total protein level to baseline compared to vehicle (figure 3B, P<0.01). IgM levels remained low after LPS instillation throughout the course of inflammation (figure 3C). No differences between BIRB 796 and vehicle treatment occurred. Interestingly, during LTA-induced inflammation the increase in BAL fluid IgM levels were abolished by BIRB 796 treatment (figure 3D, P<0.05).

### BIRB 796 and coagulation

Previously, it was shown that BIRB 796 affects systemic activation of the coagulation system in humans challenged with LPS intravenously [23]. Here we evaluated coagulation activation in BAL fluid by measuring the concentrations of TATc (by ELISA) and D-dimer (by fibrinogen western blots, where the 200 kD band represents D-dimer)(figure 4).

LPS instillation did not result in detectable D-dimer levels in BAL fluid at the 3 and 6 hour time points. After 18 hours of LPS-induced inflammation D-dimer levels could be detected; BIRB 796 treatment did not statically significantly alter D-dimer levels (figure 4A and B). TATc levels increased in response to LPS; BIRB 796 administration resulted in higher TATc levels in BAL fluid at 18 hours (P<0.05; figure 4C). A trend towards enhanced TATc levels after BIRB 796 treatment was already found at 6 hours.

In LTA-induced inflammation D-dimer levels were detectable at the 6 hour time point (figure 4D and E). BIRB 796 treatment resulted in enhanced D-dimer levels compared to the vehicle.

### Table 2: Lavage fluid levels of inflammatory mediators

<table>
<thead>
<tr>
<th></th>
<th>TNF-α</th>
<th>IL-6</th>
<th>KC</th>
<th>MIP-2</th>
<th>LIX</th>
</tr>
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<tbody>
<tr>
<td>0 hours Sham</td>
<td>0.01 ± 0.01</td>
<td>N.D.</td>
<td>0.23 ± 0.04</td>
<td>0.82 ± 0.14</td>
<td>1.48 ± 0.34</td>
</tr>
<tr>
<td>3 hours LPS Vehicle</td>
<td>10.62 ± 0.85</td>
<td>1.34 ± 0.19</td>
<td>4.40 ± 0.49</td>
<td>1.94 ± 0.26</td>
<td>2.23 ± 0.33</td>
</tr>
<tr>
<td>BIRB</td>
<td>6.42 ± 0.60**</td>
<td>0.85 ± 0.07*</td>
<td>3.99 ± 0.49</td>
<td>1.44 ± 0.12*</td>
<td>1.59 ± 0.27</td>
</tr>
<tr>
<td>6 hours LPS Vehicle</td>
<td>4.60 ± 0.37</td>
<td>0.77 ± 0.07</td>
<td>0.79 ± 0.04</td>
<td>0.65 ± 0.03</td>
<td>0.60 ± 0.07</td>
</tr>
<tr>
<td>BIRB</td>
<td>2.79 ± 0.56*</td>
<td>0.62 ± 0.13</td>
<td>0.59 ± 0.14</td>
<td>0.47 ± 0.08*</td>
<td>N.D.*</td>
</tr>
<tr>
<td>18 hours LPS Vehicle</td>
<td>0.96 ± 0.09</td>
<td>0.36 ± 0.04</td>
<td>0.37 ± 0.03</td>
<td>0.48 ± 0.05</td>
<td>2.67 ± 0.27</td>
</tr>
<tr>
<td>BIRB</td>
<td>0.26 ± 0.06**</td>
<td>0.38 ± 0.09</td>
<td>0.38 ± 0.04</td>
<td>0.57 ± 0.03</td>
<td>1.41 ± 0.21**</td>
</tr>
<tr>
<td>6 hours LTA Vehicle</td>
<td>0.09 ± 0.01</td>
<td>0.43 ± 0.08</td>
<td>0.35 ± 0.04</td>
<td>0.62 ± 0.06</td>
<td>0.84 ± 0.12</td>
</tr>
<tr>
<td>BIRB</td>
<td>0.13 ± 0.03</td>
<td>0.23 ± 0.07</td>
<td>0.55 ± 0.11</td>
<td>0.75 ± 0.06</td>
<td>1.56 ± 0.17**</td>
</tr>
</tbody>
</table>

BAL fluid inflammatory mediator levels (ng/ml). Mice were inoculated with LPS (1 µg) or LTA (100 µg) and simultaneously treated with either BIRB 796 (10 mg/kg) or vehicle. After 3, 6 and 18 hours of LPS and 6 hours of LTA inflammation animals were sacrificed; sham samples were obtained from naïve mice. Data are expressed as mean SEM, N=4-8. * = P < 0.05; ** = P < 0.01 vs. vehicle.
Figure 4: Local p38 MAPK inhibition increases TATc levels after LPS instillation and D-dimers levels after LTA administration. Mice were inoculated with LPS (1 μg) or LTA (100 μg) and simultaneously treated with either BIRB 796 (10 mg/kg) or vehicle. After 3, 6 and 18 hours of LPS and 6 hours of LTA inflammation animals were sacrificed; sham samples were obtained from naïve mice. BAL fluid obtained from the mice experiments D-dimers levels were determined by fibrinogen western blot and TATc levels were measured by ELISA. (A, B) Western blot for fibrinogen and TATc (C) after LPS administration. (D, E) Fibrinogen blots and TATc levels (F) in BAL cells after LTA administration. Data are expressed as means ± SEM (n = 4-8) * = P < 0.05
control (figure 4E, P < 0.05). TATc levels also showed an increase in response to LTA, however no effects of BIRB 796 were observed (figure 4F).

Discussion

The inflammatory response is pivotal in host-defense against invading pathogens. However, hyper-inflammation is unwanted as it may be detrimental to the host [7]. Here we studied the impact of direct intrabronchial p38 MAPK inhibition on lung inflammatory responses induced by LPS or LTA administration via the airways. Our approach of intrapulmonary treatment was chosen considering that this route of administration potentially could avoid unneeded or unwanted systemic anti-inflammatory effects.

In in vitro experiments, BIRB 796 reduced p38 MAPK phosphorylation in response to LPS or LTA in all cell types relevant for lung inflammation (i.e. PMNs, macrophages and epithelial cells), resulting in reduced production of cytokines or chemokines. PMNs displayed p38 MAPK phosphorylation very shortly after exposure to LPS. This could either be due to enhanced reactivity to LPS or due to possible activation during the isolation process. Notably, cells were not pre-incubated with BIRB 796; thus, not only is the phosphorylation of p38 MAPK nigh immediate, the inhibition of this process by BIRB 796 is equally rapid. Epithelial and macrophages cell lines exhibited a comparable reduction in p38 MAPK phosphorylation. Interestingly, the strongest reduction in cytokine/chemokine production was observed in the macrophage cell line. The underlying mechanism to this discrepancy could be that p38 MAPK directly stabilizes TNF-α mRNA, which has not been described for KC [24]. In line with our findings, p38 MAPK inhibition did not impact IL-8 production in primary human epithelial cells [25]. Thus, overall BIRB 796 reduces the inflammatory response to LPS (or LTA) in important pulmonary cells lines.

Profound differences between pulmonary inflammation induced by LPS and by LTA are apparent from our data. Although in both settings p38 MAPK phosphorylation was elicited, weaker effects on chemotaxis to the lung compartment and inflammatory mediator production were observed in LTA-induced inflammation. Similar observations were made by our laboratory in a human model of intrapulmonary LPS and LTA delivery [26]. Still, it remains striking that in LTA-induced inflammation IgM, protein and D-dimer levels were greatly enhanced, while inflammatory mediators were not strongly induced when compared to the responses induced by LPS. LPS and LTA activate cells via distinct receptors (TLR4 and TLR2 respectively), in part using different adaptor proteins (Myeloid differentiation primary response gene 88(MyD88)/TIR-domain-containing adapter-inducing interferon-β(TRIF) and MyD88 respectively) [4]. More research is warranted to establish via which routes and via which cell types LPS and LTA elicit different inflammatory and procoagulant responses in the lungs.

In human experimental endotoxemia, systemic application of BIRB 796 reduced p38 MAPK phosphorylation [21]. We here show that local administration of BIRB 796, directly into the airways, also inhibits p38 MAPK phosphorylation in response to both LPS- and LTA-induced
Intrapulmonary p38 inhibition partially prevents inflammation in mice. It should be noted that these observations were done in BAL cells and do not provide insight on effects on lung epithelial cells. Moreover, at both 3 and 6 hours p38 MAPK phosphorylation was reduced, while the dominate cell type, PMNs, are of extra pulmonary origin. The inhibition of p38 MAPK in newly arriving PMNs could be due to either lack of activation by lung residents or to sustained BIRB 796 presence in the pulmonary compartment at these time points.

While we did not find effects on PMN recruitment into the bronchoalveolar space, other studies did describe effects of p38 MAPK inhibition on cell influx. Orally administered SB239063 diminished eosinophilia in murine ovalbumin-induced allergic lung inflammation [16]. Also, LPS-induced pulmonary inflammatory responses in guinea pigs were reduced by this compound [17]. Another oral drug, M39, inhibited pulmonary PMN accumulation in LPS instilled mice [18]. Intraperitoneal treatment with SB203580 reduced PMN recruitment into the bronchoalveolar space following intratracheal LPS administration in mice [19]. Moreover, in a model of systemic inflammation elicited by Streptococcus pyogenes M1 protein inhibition of p38 MAPK (by intraperitoneal administration of either SKF 86002 or SB 239063) reduced pulmonary edema and PMN influx [27]. Differences in outcome with our study could be explained by different administration routes. Systemic treatment, either through oral or intraperitoneal administration, probably also exerts effects on cells outside the focus of inflammation, influencing PMN influx primarily by an effect on circulating cells.

Local BIRB 796 administration did affect inflammatory mediator levels in BAL fluid. TNF-α levels were strongly reduced by BIRB 796 treatment after intrapulmonary delivery of LPS at all time points examined. This is in accordance with previous studies demonstrating reduced lung TNF-α levels after systemic inhibition of p38 MAPK [28,29]. Local BIRB 796 administration also diminished IL-6 levels in BAL fluid after LPS instillation. These findings are in line with our earlier investigation in healthy humans, in whom oral BIRB 796 strongly attenuated TNF-α and IL-6 release after intravenous injection of LPS [21]. Remarkably, the effect of BIRB 796 on the release of the PMN attracting chemokine LIX [30], a murine analog of CXCL5, depended on the stimulus: whereas LIX levels were reduced by BIRB 769 6 and 18 hours after LPS administration, BIRB 796 treated mice displayed increased LIX concentrations 6 hours after LTA instillation. Of note, LIX is exclusively produced by epithelial cells [31]; therefore these data suggest that either BIRB 769 differentially affects the respiratory epithelium after LPS or LTA stimulation and/or that the effects of p38 MAPK inhibition on LIX release during LPS-induced inflammation are partially mediated indirectly, via inhibition of the release of proinflammatory cytokines such as TNF-α by cell types other than epithelial cells. BIRB 796 did not impact KC levels in vivo. Reported effects of p38 MAPK inhibition on KC production are diverse: down regulation [29] and no effects [18] have both been described.

It has been suggested that p38 MAPK inhibition enhances epithelial barrier functions [29,32]. We here determined total protein and IgM levels in BAL fluid as markers for integrity of the lung epithelial barrier [33]. After LPS administration protein and IgM levels were increased after 18 hours, with no differences between BIRB 796 and vehicle treated mice. BIRB 796 did show some protective effect on barrier integrity in the LPS model, as reflected by reduced
protein concentrations in BAL fluid at 3 hours, although at this early time point protein or IgM levels did not exceed those in control mice. LTA administration clearly was associated with protein leak, as indicated by elevated protein and IgM levels in BAL fluid at 6 hours. Strikingly, in the LTA model BIRB 796 reduced total protein and IgM to near baseline levels. Notably, since BIRB 796 did not influence PMN numbers, the mechanism by which local BIRB 796 exerts protective effects on barrier integrity is PMN independent.

In human endotoxemia, p38 MAPK inhibition reduced activation of coagulation [23]. In accordance, a recent study demonstrated p38 MAPK's control on prothrombin expression by RNA 3' end process regulation [34]. Much to our surprise, we here observed an opposite effect of locally administered BIRB 796: in both the LPS and LTA models, BIRB 796 enhanced rather than attenuated local coagulation activation, as reflected by elevated levels of TATc and D-dimer in BAL fluid respectively. At present, the mechanisms involved in this paradoxical effect of administration of BIRB 769 via the airways on pulmonary coagulation are elusive.

To the best of our knowledge, the present study is the first to investigate the effects of a p38 MAPK inhibitor administered directly into the lungs. Local application of p38 MAPK inhibitors may result in stronger effects at sites of inflammation, at the same time circumventing undesired systemic effects. We showed that although local administration of BIRB 796 prevents p38 MAPK phosphorylation and reduces inflammatory mediator levels in LPS-induced lung inflammation, PMN recruitment remains unaltered and coagulation activation is even enhanced. These results taken together with earlier studies on systemic administration of p38 MAPK inhibitors in rodents and humans cited above suggest that direct intrapulmonary delivery of a p38 MAPK inhibitor is less effective in inhibiting inflammation and is associated with unexpected procoagulant effects in the bronchoalveolar space.

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Intrapulmonary p38 inhibition partially prevents inflammation.

References


Intrapulmonary p38 inhibition partially prevents inflammation


