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RESEARCH PAPER

Therapeutic inhibition of CXCR2 by Reparixin attenuates acute lung injury in mice

A Zarbock[1,2], M Allegretti[3] and K Ley[1]

[1]*Division of Inflammation Biology, La Jolla Institute for Allergy and Immunology, La Jolla, CA, USA;* [2]*Department of Anesthesiology and Intensive Care Medicine, University of Muenster, Muenster, Germany and* [3]*Research Centre, Dompé pha.r.ma s.p.a., L'Aquila, Italy*

Background and purpose: Acute lung injury (ALI) remains a major challenge in critical care medicine. Both neutrophils and chemokines have been proposed as key components in the development of ALI. The main chemokine receptor on neutrophils is CXCR2, which regulates neutrophil recruitment and vascular permeability, but no small molecule CXCR2 inhibitor has been demonstrated to be effective in ALI or animal models of ALI. To investigate the functional relevance of the CXCR2 inhibitor Reparixin *in vivo*, we determined its effects in two models of ALI, induced by either lipopolysaccharide (LPS) inhalation or acid instillation.
Experimental approach: In two ALI models in mice, we measured vascular permeability by Evans blue and evaluated neutrophil recruitment into the lung vasculature, interstitium and airspace by flow cytometry.
Key results: Pharmacological inhibition of CXCR2 by Reparixin reduced CXCL1-induced leukocyte arrest in the microcirculation of the cremaster muscle, but did not influence arrest in response to leukotriene B_4 (LTB_4) demonstrating specificity. Reparixin (15 $\mu g\,g^{-1}$) reduced neutrophil recruitment in the lung by approximately 50% in a model of LPS-induced ALI. A higher dose did not provide additional reduction of neutrophil recruitment. This dose also reduced accumulation of neutrophils in the interstitial compartment and vascular permeability in LPS-induced ALI. Furthermore, both prophylactic and therapeutic application of Reparixin improved gas exchange, and reduced neutrophil recruitment and vascular permeability in a clinically relevant model of acid-induced ALI.
Conclusions and implications: Reparixin, a non-competitive allosteric CXCR2 inhibitor attenuates ALI by reducing neutrophil recruitment and vascular permeability.
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Keywords: acute lung injury; CXCR2; inflammation; leukocyte; permeability

Abbreviations: ALI, acute lung injury; BAL, bronchoalveolar lavage; IL-8, interleukin 8; i.p., intraperitoneal; LPS, lipopolysaccharide; LTB_4, leukotriene B_4

Introduction

Acute lung injury (ALI) is a clinical syndrome characterized by an excessive inflammatory response to both extra- and intrapulmonary insults (Ware and Matthay, 2000). During the development of ALI, the alveolar-capillary integrity is damaged, leading to neutrophil infiltration, interstitial and alveolar oedema, and diminished gas exchange (Ware and Matthay, 2000). ALI is associated with a high morbidity and mortality despite improved ventilation strategies (Levitt and Matthay, 2006). As yet, there are no specific treatments for ALI available.

Correspondence: Dr K Ley, Division of Inflammation Biology, La Jolla Institute for Allergy and Immunology, 9420 Athena Circle Drive, La Jolla, CA 92037, USA.
E-mail: klaus@liai.org
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Accumulation and recruitment of polymorphonuclear leukocytes in the lung are key events in the development of ALI (Ware and Matthay, 2000). Neutrophil recruitment in the lung proceeds in a cascade-like fashion of activation, intravascular accumulation and transendothelial and transepithelial migration (Reutershan *et al*., 2005). Depending on the underlying cause of ALI, different adhesion molecules and chemokines are involved in neutrophil recruitment (Doerschuk, 2001). CXCR2, the chemokine receptor for CXCL1 (keratinocyte-derived chemokine) and CXCL2/3 (macrophage inflammatory protein 2) in mice, is critically involved in neutrophil recruitment and the regulation of vascular permeability in different models of ALI (Belperio *et al*., 2002; Reutershan *et al*., 2006). CXCR2 on hematopoietic and non-hematopoietic cells each contributes about equally to neutrophil recruitment into the lung following lipopolysaccharide (LPS) inhalation (Reutershan *et al*., 2006).

Elimination of CXCR2 in both cell types completely blocks neutrophil recruitment into the alveolar space in response to inhaled LPS (Reutershan *et al*., 2006). Human CXCL8 (interleukin 8; IL-8), a ligand for human CXCR2 (Zeilhofer and Schorr, 2000) and CXCR1 (Zeilhofer and Schorr, 2000), is also associated with the development and outcome of ALI in patients (Krupa *et al*., 2004). A recently published study identified mouse CXCR1 (Fan *et al*., 2007). Murine CXCR1 shares 68% amino-acid identity with human CXCR1 and 66% with mouse CXCR2. mCXCR1 is predominantly expressed in lung and leukocyte-rich tissues (Fan *et al*., 2007). When transfected into BaF3 pro-B cells, mCXCR1 supports binding of mCXCL6 and hCXCL8, both of which lead to GTPγS exchange. The transfected cells migrate towards CXCL6 with an optimal activity at 1 μM.

CXCR2 is a 7-transmembrane G protein-coupled receptor (Olson and Ley, 2002). Following engagement of this receptor, the Gβγ-complex dissociates from the $G\alpha_i$-subunit and can activate phosphoinositide-3 kinase, different subtypes of phospholipase C and P-Rex-1 (Camps *et al*., 1992; Hirsch *et al*., 2000; Welch *et al*., 2002). The downstream effectors of these molecules initiate a broad range of functional responses, including arrest from rolling (Zarbock *et al*., 2007a), cytoskeletal rearrangement, cell polarization, chemotaxis, degranulation and respiratory burst (Bokoch, 1995).

G-protein-coupled receptors are targets for the development of new strategies to control undesired inflammatory responses and the pathogenesis of diseases (Proudfoot, 2002). These strategies include N-terminally modified chemokines, antibodies and small-molecule antagonists. Reparixin, a non-competitive allosteric inhibitor of CXCR1/2, also known as Repertaxin, specifically blocks CXCR1/2-mediated mouse and human neutrophil migration *in vitro* without affecting other receptors (Bertini *et al*., 2004). Reparixin inhibits CXCL8-induced neutrophil activation through human CXCR1 and human CXCR2 and blocks phosphorylation of downstream signalling molecules. Reparixin prevents the increase of intracellular free calcium, elastase release and production of reactive oxygen intermediates (Bertini *et al*., 2004), but leaves phagocytosis of *Escherichia coli* bacteria unaffected (Casilli *et al*., 2005). The application of Reparixin showed beneficial effects in a bacteria-induced peritonitis model (Bertini *et al*., 2004), a venom-induced lung injury model (Coelho *et al*., 2007) and different models of ischaemia-reperfusion injury (Bertini *et al*., 2004; Souza *et al*., 2004; Cavalieri *et al*., 2005; Cugini *et al*., 2005; Garau *et al*., 2005).

In the present study, we investigated the therapeutic potential of Reparixin in murine models of LPS-induced pulmonary inflammation and acid-induced ALI. The LPS model is a milder model of pulmonary inflammation in which neutrophil recruitment and vascular permeability can be examined. The acid-induced lung injury is a clinically relevant model in which functional parameters including the arterial oxygen partial pressure can be measured. In order to investigate the specificity of Reparixin for mouse CXCR2 *in vivo*, intravital microscopy of the cremaster muscle was performed and leukocyte arrest was assessed.

Materials and methods

Animals

The Animal Care and Use Committee of the University of Virginia (Charlottesville) approved all animal experiments. We used 8–12 weeks old C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME, USA). Mice were housed in a barrier facility under specific pathogen-free conditions.

Surgical preparation and intravital microscopy

Mice were anaesthetized with an intraperitoneal (i.p.) injection of ketamine hydrochloride (125 mg kg^{-1}; Sanofi Winthrop Pharmaceuticals, New York, NY, USA), atropine sulphate (0.025 mg kg^{-1}; Fujisawa, Deerfield, IL, USA) and xylazine (12.5 mg kg^{-1}; Tranqui Ved; Phonix Scientific, San Marcos, CA, USA) and placed on a heating pad to maintain body temperature. For specificity testing, the cremaster muscle was prepared after tracheal intubation and cannulation of the carotid artery for intravital microscopy as previously described (Zarbock *et al*., 2007b). Microscopic observations were made on postcapillary venules (diameter 20–40 μm) using an intravital microscope (Axioskop; Zeiss, Thornwood, NY, USA) with a saline immersion objective (SW 40/0.75 numerical aperture). A CCD camera (model VE-1000CD, Dage-MTI) was used for recording. Leukocyte arrest was determined before and 1 min after intravenous (i.v.) injection of 600 ng CXCL1 (PeproTech, Rocky Hill, NJ, USA) or 5 μg leukotriene B_4 (LTB_4; Cayman Chemical, Ann Arbor, MI, USA) as described previously (Zarbock *et al*., 2007a). Arrest was defined as leukocyte adhesion longer than 30 s and expressed as number of cells per surface area. Surface area, *S*, was calculated for each vessel using $S = \pi \times d \times l_v$, where *d* is the diameter and l_v is the length of the vessel. Blood flow centreline velocity was measured using a dual photodiode sensor and a digital online cross-correlation program (Circusoft Instrumentation, Hockessin, DE, USA). Centreline velocity and wall shear stress were calculated as described previously (Lipowsky and Zweifach, 1978; Long *et al*., 2004).

Murine model of LPS-induced pulmonary inflammation and acid-induced ALI

LPS-induced pulmonary inflammation was induced as previously described (Reutershan *et al*., 2007). Briefly, mice were exposed to aerosolized LPS from *Salmonella enteritidis* (500 μg mL^{-1}, Sigma-Aldrich, St Louis, MO, USA) for 30 min and analysed 24 h later. Aerosolized saline served as a negative control.

Acid-induced ALI was induced as previously described (Zarbock *et al*., 2006). Briefly, 2 μL g^{-1} body weight of HCl with a pH of 1.5 was injected intratracheally, followed by a bolus of air (30 μL g^{-1}). After the induction of ALI, the trachea of each mouse was intubated with a tube (PE 90) and the mouse was ventilated with a respirator (MiniVent, Type 845; Hugo Sachs Elektronik) for 2 h (tidal volume, 10 μL g^{-1}; respiration rate, 140 per min; fraction of inspiratory oxygen (FiO_2), 0.21). Control animals received saline instead of HCl in the same manner.

Reparixin (*R*(–)-2-(4-isobutylphenyl)propionyl methansulphonamide) as the L-lysine salt (Bertini *et al*., 2004) was obtained from Dompé pha.r.ma (Italy) and dissolved in saline. Where indicated, Reparixin was injected i.p. at 15 min before and 2 h after LPS-induced pulmonary inflammation. In the model of acid-induced ALI, Reparixin was injected i.p. either 15 min before or 15 min after the induction of ALI.

Neutrophil recruitment into the lung

After the mice were killed, bronchoalveolar lavage (BAL) was collected (5×1 mL phosphate-buffered saline (PBS)). BAL fluid was centrifuged and neutrophils were counted using Kimura stain.

Intravascular and interstitial neutrophils in the lungs were distinguished by a flow-cytometry-based method as previously described (Zarbock *et al*., 2006). Briefly, an Alexa 633-labelled GR-1 antibody (clone RB6-8C5, staining kit: Invitrogen Corp., Carlsbad, CA, USA) was injected i.v. 5 min, before death. This labels only intravascular neutrophils. After performing BAL, the inferior vena cava was dissected and non-adherent neutrophils were removed from the pulmonary vasculature by flushing 10 mL of PBS at 25 cm H_2O through the spontaneously beating right ventricle. Lungs were removed, minced and digested with enzyme cocktail at 37 °C for 60 min. In order to produce a cell suspension, the digested lungs were passed through a 70 μm cell strainer (BD Falcon, Bedford, MA, USA), erythrocytes were lysed and the remaining leukocytes were counted. The fraction of neutrophils in the suspension was determined by flow cytometry (FACS Calibur; Becton Dickinson, San Jose, CA, USA). Neutrophils were identified by their typical appearance in the forward/sideward scatter and their expression of CD45 (clone 30-F11), 7/4 (clone 7/4; both BD Biosciences-Pharmingen, San Diego, CA, USA) and GR-1 (clone RB6-8C5). The i.v. injected labelled GR-1 Ab was used to differentiate between intravascular ($CD45^+7/4^+GR\text{-}1^+$) and interstitial ($CD45^+7/4^+GR\text{-}1^-$) neutrophils.

In acid-induced ALI, vascular permeability was determined by measuring protein concentration in the supernatant (Lowry's method).

Pulmonary microvascular permeability

Pulmonary microvascular permeability in LPS- and saline-treated mice pretreated with Reparixin or vehicle was determined by Evans blue dye extravasation as described previously (Reutershan *et al*., 2006). Briefly, Evans blue (20 mg kg^{-1}; Sigma-Aldrich) was injected i.v. 30 min prior to death. Lungs were perfused, removed and Evans blue was extracted. The absorption of Evans blue was measured, corrected for haemoglobin and calculated in the different animal groups, 6 h after LPS or saline inhalation.

Pulmonary function: oxygenation

Blood was obtained from an arterial catheter to measure arterial blood gas (Rapidlab 800 System; Bayer HealthCare). Partial pressure of arterial oxygen (PaO_2) was normalized to the fraction of inspiratory oxygen (FiO_2) to obtain the oxygenation index.

Statistics

Statistical analysis was performed with SPSS (version 9.0, Chicago, IL, USA) and included one-way analysis of variance, Student–Newman–Keuls test and *t*-test where appropriate. All data are presented as mean ± s.e.m.; $P<0.05$ was considered significant.

Results

Reparixin, a non-competitive allosteric CXCR2 inhibitor, specifically blocks CXCL1-induced leukocyte arrest in vivo

It has been shown that Reparixin reduces ligand binding to human CXCR1 and CXCR2, calcium influx and downstream signalling in response to human CXCL8 and neutrophil recruitment into the liver in a mouse model of ischaemia-reperfusion injury (Bertini *et al*., 2004). In order to investigate whether Reparixin specifically blocks CXCR2-induced leukocyte arrest in mice, we performed intravital microscopy of the cremaster muscle and investigated leukocyte arrest in response to CXCL1 and LTB_4. LTB_4, which binds to and activates the $G\alpha_i$-coupled receptor BLTR1 (Wettschureck and Offermanns, 2005) was used as a positive control. Mice pretreated with Reparixin showed the same number of adherent leukocytes under baseline conditions compared to control mice (Figure 1a). Injection of the recombinant murine chemokine CXCL1 induced immediate firm arrest in control mice (Figure 1a). The number of adherent cells per area in mice pretreated with Reparixin was significantly reduced after CXCL1 injection (Figure 1a). The treatment with Reparixin did not influence leukocyte adhesion in response to LTB_4 (Figure 1b). The venules were of similar size and had similar haemodynamic parameters (data not shown). These data suggest that inhibition of the CXCR2 receptor by Reparixin selectively blocked CXCR2-induced arrest without inhibiting other G-protein-coupled receptors.

Efficiency of Reparixin in inhibiting neutrophil recruitment in the lung

Reparixin at concentrations of 3 μg g^{-1} and 15 μg g^{-1} reduced neutrophil recruitment and liver damage by approximately 30% and 80% in a model of ischaemia-reperfusion injury, respectively (Bertini *et al*., 2004). In order to investigate the necessary dosage of Reparixin to inhibit neutrophil recruitment in the inflamed lung, we tested the effects of Reparixin at 3, 15 and 30 μg g^{-1} on neutrophil recruitment into the alveolar compartment following LPS inhalation. Twenty-four hours after LPS exposure, neutrophil recruitment into the alveolar compartment significantly increased in control mice (Figure 2). Treatment with 3 μg g^{-1} Reparixin did not reduce neutrophil recruitment into the lung, whereas 15 μg g^{-1} led to a significant reduction in neutrophil recruitment into the alveolar space (Figure 2). The increase of the Reparixin dose to 30 μg g^{-1} did not further reduce neutrophil accumulation in BAL. Therefore, 15 μg g^{-1} of Reparixin was used in all further experiments.

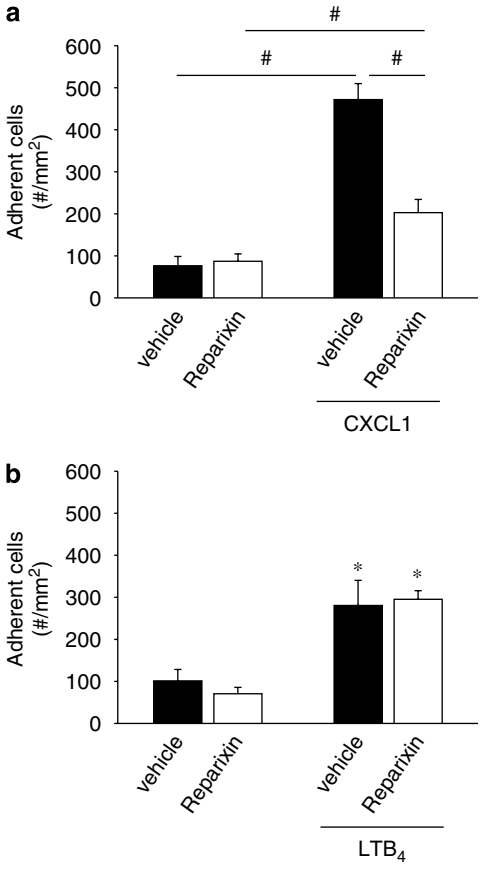

Figure 1 Effect of Reparixin on CXCL1-induced arrest from rolling *in vivo*. (**a**, **b**) Number of adherent leukocytes in cremaster muscle postcapillary venules of wild-type mice pretreated with the CXCR2 inhibitor Reparixin ($n=4$) and control mice ($n=4$) before (left) and after (right) injection with 600 ng CXCL1 (**a**) or 5 μg LTB_4 (**b**). Data presented are the mean ± s.e.m. from four mice. $^{\#}P<0.05$. $^{*}P<0.05$ compared to corresponding control.

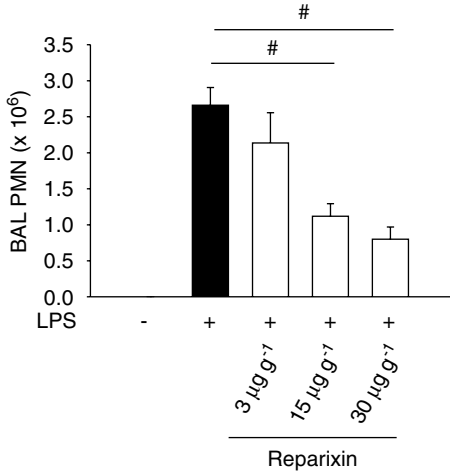

Figure 2 Dose-dependent inhibition of neutrophil recruitment into the lung following lipopolysaccharide (LPS) inhalation. Mice inhaled LPS for 30 min, and neutrophil accumulation in bronchoalveolar lavage (BAL; BAL PMN) was analysed at 24 h. Other mice were pretreated with different doses of Reparixin ($n=4$). Mean ± s.e.m. $^{\#}P<0.05$ vs LPS-treated mice ($n=4$).

Pharmacological inhibition of CXCR2 reduces transendothelial and transepithelial migration, but not intravascular accumulation of neutrophils in the microcirculation of the lung

In the lung, neutrophils first accumulate in the vasculature, followed by infiltration into the interstitial space and exit into BAL (Reutershan *et al*., 2005). In order to investigate which step in the neutrophil recruitment cascade was affected by the inhibition of CXCR2, mice received a dose of 15 μg g^{-1} Reparixin 15 min before and 2 h after LPS inhalation. Reparixin did not affect the number of neutrophils in the intravascular (Figure 3a), interstitial (Figure 3b)

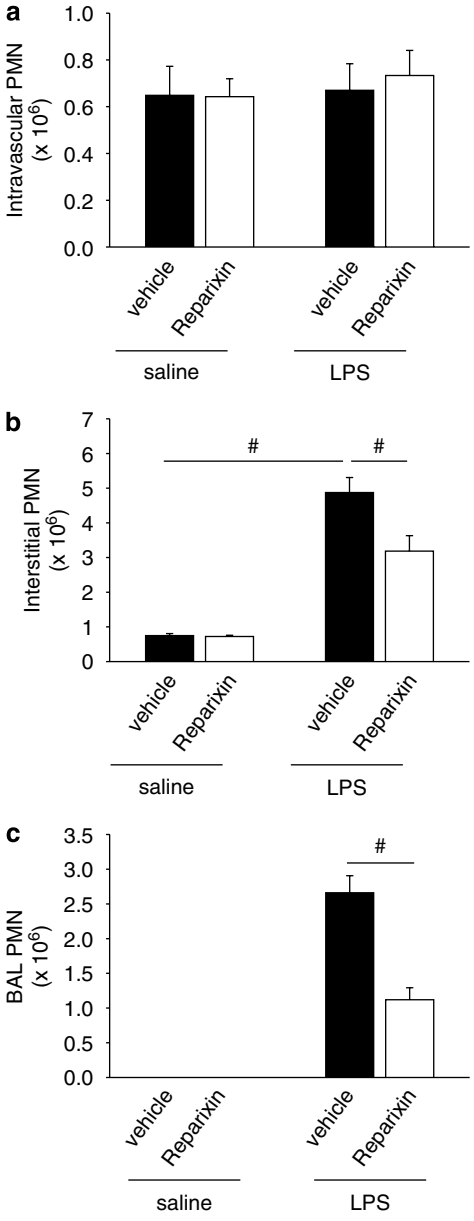

Figure 3 Neutrophil recruitment into the lung in a model of lipopolysaccharide (LPS)-induced pulmonary inflammation. Neutrophil recruitment into the different compartments of the lung was measured in Reparixin-treated (15 μg g^{-1}) mice ($n=4$) and control mice ($n=4$) by flow cytometry 24 h after LPS inhalation. Neutrophil accumulation in the intravascular (**a**), interstitial (**b**) and alveolar (**c**) compartments of Reparixin-treated mice and control mice. $^{\#}P<0.05$.

or alveolar compartments (Figure 3c) under baseline conditions compared to vehicle-treated control mice. After LPS exposure, Reparixin significantly reduced neutrophil numbers in the interstitial (Figure 3b) and alveolar compartments (Figure 3c). These data show that CXCR2 is not involved in neutrophil accumulation in the microcirculation of the lung under normal conditions, but this receptor is critical for neutrophil migration into other compartments. The stronger inhibition seen in BAL compared to the interstitial space suggests that inhibition of both transendothelial and transepithelial migration contributes to this effect.

Pulmonary microvascular permeability is reduced by CXCR2 blockade

In addition to neutrophil infiltration, vascular leakage is also critically involved in pulmonary inflammation and ALI. We therefore tested the role of Reparixin in LPS-induced microvascular permeability. In control mice, LPS inhalation caused a significant increase of the pulmonary vascular permeability as measured by Evans blue (Figure 4). This vascular permeability increase was reduced by approximately 65% in mice pretreated with Reparixin (Figure 4).

Reparixin protects against acid-induced acute lung injury

Based on our finding that the inhibition of CXCR2 by Reparixin reduces neutrophil recruitment and vascular permeability in response to LPS exposure, we hypothesized that this inhibitor may also improve functional and morphological parameters in a clinically relevant model of acid-induced ALI (Zarbock *et al*., 2006). Two hours after induction of ALI with HCl, ventilated control mice showed reduced gas exchange (Figure 5a), increased neutrophil numbers in BAL (Figure 5b) and elevated protein levels in BAL (Figure 5c) compared to mice injected with saline. Application of Reparixin 15 min before the induction of ALI, significantly improved the gas exchange (Figure 5a), but did not affect that found in mice injected with saline. Reparixin treatment reduced the number of neutrophils in the alveolar space (Figure 5b), but the inhibition was not complete. The protein concentration in the BAL of control mice significantly increased 2 h following injecting HCl. Injection of Reparixin 15 min after the induction of ALI diminished the protein concentration in BAL compared to vehicle-treated control mice (Figure 5c).

As CXCR2 inhibition by Reparixin reduced neutrophil recruitment into BAL 2 h after induction of acid-induced ALI,

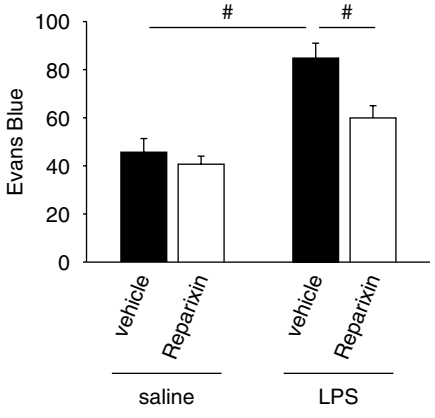

Figure 4 Effect of Reparixin on pulmonary microvascular permeability. Pulmonary permeability measured by Evans blue extravasation was determined in Reparixin-treated ($n=4$) and control mice ($n=4$) 6 h after lipopolysaccharide (LPS) exposure. $^{\#}P<0.05$.

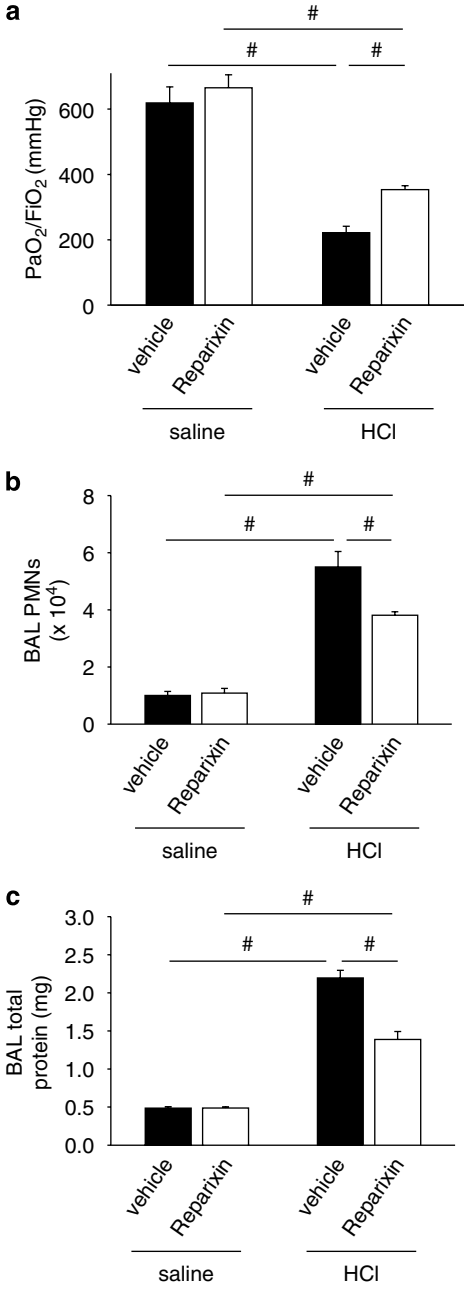

Figure 5 Reparixin protects from acid-induced acute lung injury (ALI). Two hours after intratracheal saline or HCl application, gas exchange (**a**), neutrophils in bronchoalveolar lavage (BAL) fluid (**b**) and vascular permeability (**c**) were determined in Reparixin-treated (15 $\mu g\,g^{-1}$; open bars, $n=4$ mice) and control mice (black bars, $n=4$ mice). $^{\#}P<0.05$.

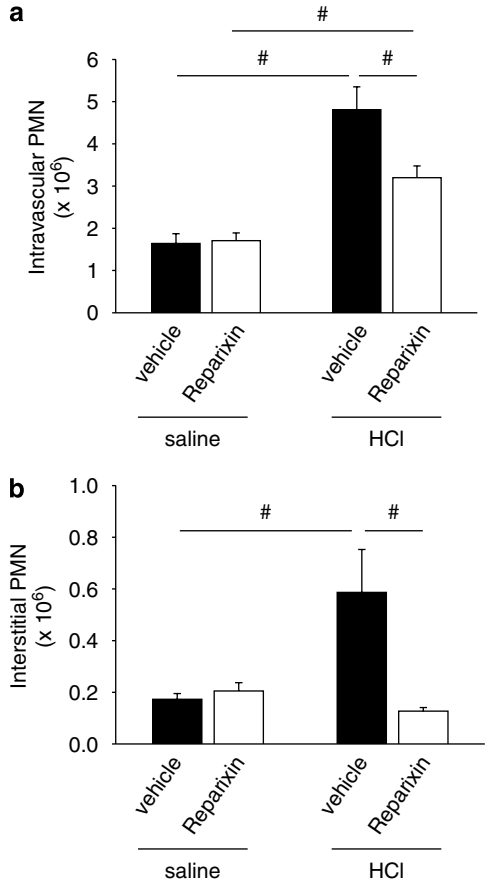

Figure 6 Neutrophil recruitment into the lung following the induction of the acid-induced acute lung injury (ALI). Neutrophil recruitment into the different compartments of the lung was measured in Reparixin-treated (15 $\mu g\,g^{-1}$) mice ($n=4$) and control mice ($n=4$–6) by flow cytometry 2 h after saline or HCl application. Neutrophil accumulation was measured in the intravascular (**a**) and interstitial (**b**) compartments of Reparixin-treated mice and control mice. $^{\#}P<0.05$.

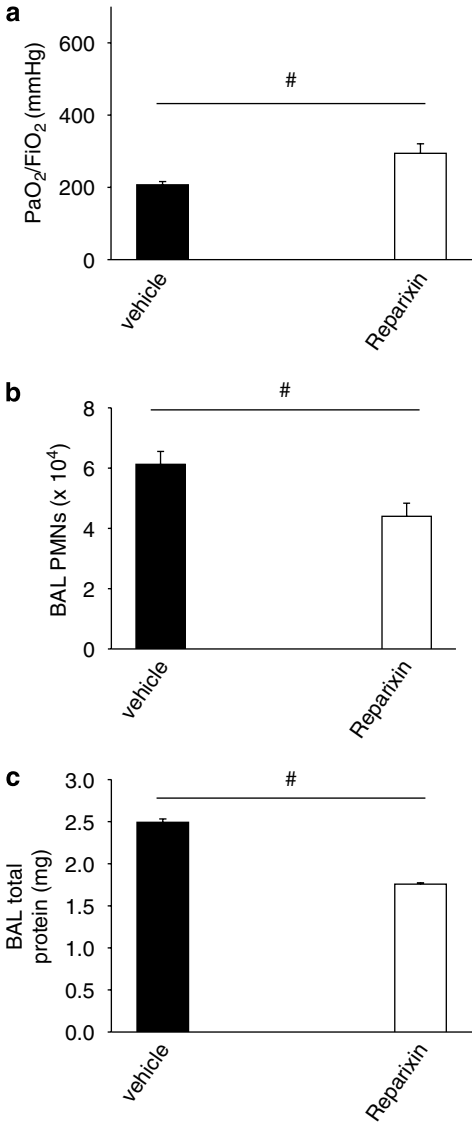

Figure 7 Therapeutic application of Reparixin attenuates acid-induced acute lung injury. Two hours after initiation of acid-induced acute lung injury (ALI), gas exchange (**a**), neutrophils in bronchoalveolar lavage (BAL) fluid (**b**) and vascular permeability (**c**) were determined in mice that received Reparixin (15 $\mu g\,g^{-1}$, $n=4$) at 15 min after induction of ALI and in control mice with ALI but no Reparixin ($n=4$). $^{\#}P<0.05$.

we investigated which step in the neutrophil recruitment cascade was influenced by CXCR2 inhibition. The number of neutrophils in the intravascular (Figure 6a) and interstitial (Figure 6b) compartments significantly increased in control mice 2 h after intratracheal instillation of HCl. After induction of acid-induced ALI, Reparixin significantly reduced the numbers of neutrophils in the intravascular (Figure 6a) and interstitial compartments (Figure 6b).

Therapeutic application of Reparixin attenuates acid-induced acute lung injury

In order to investigate whether Reparixin improves functional and morphological parameters of ALI even when given after the insult, we injected Reparixin 15 min after the induction of the acid-induced lung injury and measured gas exchange, neutrophil recruitment and protein concentration in BAL. Although Reparixin is a non-competitive allosteric inhibitor, application of this inhibitor in a therapeutic approach improved gas exchange (Figure 7a), and reduced neutrophil recruitment (Figure 7b) and vascular permeability (Figure 7c).

Discussion

In a murine model of LPS-induced pulmonary inflammation and acid-induced ALI, we characterized the effects of the non-competitive allosteric CXCR2 inhibitor Reparixin on neutrophil recruitment and vascular permeability. We found that Reparixin reduced transendothelial and transepithelial migration of neutrophils, but did not regulate neutrophil accumulation in the intravascular compartment in the lung in response to aerosolized LPS. In addition to these findings, we demonstrated that Reparixin protected against morphological and functional changes in a clinically relevant model of acid-induced ALI.

Previous studies have shown that Reparixin specifically blocks human and mouse neutrophil migration induced by

CXCL8 or CXCL1 *in vitro* (Bertini *et al.*, 2004). Reparixin also inhibits CD11b upregulation and neutrophil adhesion to fibrinogen (Casilli *et al.*, 2005). However, neutrophil phagocytosis of *E. coli* bacteria was unaffected (Casilli *et al.*, 2005). These results indicate that Reparixin only blocks some, but not all, CXCR1 and -2 functions. Several studies demonstrated that different mouse tissues express an mRNA encoding CXCR1 homologue (Fu *et al.*, 2005; Moepps *et al.*, 2006; Fan *et al.*, 2007). Although the cellular expression of mCXCR1 is not known, transfected cells responded to mCXCL6 (Fan *et al.*, 2007). Therefore, we cannot exclude that Reparixin might also block mCXCR1 function. However, BLTR, another $G\alpha_i$-coupled receptor was not influenced by Reparixin treatment. As Reparixin effectively blocks the neutrophil arrest response to mCXCL1, which is a ligand for CXCR2, but not mCXCR1 (Fan *et al.*, 2007), these data demonstrate that Reparixin is an efficient and specific inhibitor of CXCR2-dependent neutrophil arrest. Under unstimulated conditions, Reparixin had no effect on neutrophil accumulation in the lung microvasculature, suggesting that the mechanism of this accumulation under these circumstances does not involve chemokine-triggered arrest. Indeed, several investigators (Worthen *et al.*, 1989; Doerschuk, 2001) have proposed that mechanical factors like reduced deformability are responsible for neutrophil accumulation in lung capillaries. However, the data showing that Reparixin reduced neutrophil accumulation in the intravascular compartment and completely abolished neutrophil accumulation in the interstitial compartment in response to HCl application demonstrate that neutrophil accumulation in the intravascular compartment is partially chemokine-dependent under inflammatory conditions. Whether the reduced number of neutrophils in the interstitial compartment is due to the reduced intravascular accumulation or due to impaired emigration remains to be elucidated.

CXCR2 plays a critical role in the development of different models of ALI (Belperio *et al.*, 2002; Sue *et al.*, 2004; Strieter *et al.*, 2005; Reutershan *et al.*, 2006). Elimination of CXCR2 by gene targeting or blocking CXCR2 with an antibody reduced neutrophil recruitment into the lung, lung oedema and protein leakage (Sue *et al.*, 2004; Reutershan *et al.*, 2006). CXCR2 receptors on haematopoietic and non-haematopoietic cells are both important for neutrophil recruitment in response to LPS, each accounting for about half of neutrophil recruitment (Reutershan *et al.*, 2006). Reparixin blocked neutrophil recruitment into the alveolar compartment by 60% following LPS exposure. These data are concordant with earlier studies from our group, which showed that elimination of CXCR2 almost abolished neutrophil recruitment into the alveolar compartment and that mediators other than CXCL1 and CXCL2/3 are also involved in neutrophil migration towards BAL taken from LPS-exposed mice *in vitro* (Reutershan *et al.*, 2006). Previous work has shown a partial inhibition of neutrophil recruitment in mice heterozygous for the CXCR2 null allele (Reutershan *et al.*, 2006). This suggested a potential benefit of a pharmaceutical receptor blocker, which usually does not achieve complete blockade at realistic *in vivo* concentrations. The present data demonstrate that CXCR2 is indeed a good target for treatment of ALI. Vascular permeability is almost completely regulated by CXCR2 on non-haematopoietic cells following LPS inhalation (Reutershan *et al.*, 2006). Reparixin reduced vascular permeability by 50% following LPS-exposure. As our dose-finding studies showed that the dose used ($15\,\mu g\,g^{-1}$) was maximally effective, we conclude that Reparixin does not block all CXCR2 effects in all cells. Indeed, previous work has shown that the cellular context may influence the susceptibility of CXCR1 and -2 to Reparixin (Bertini *et al.*, 2004). The level of surface expression of the receptors may influence the susceptibility to Reparixin. Another reason for the partial inhibition of the CXCR2 effect may be due to potentially uneven distribution of Reparixin in the tissue. This would be important, as both lung microvascular endothelial cells (Reutershan *et al.*, 2006) as well as alveolar epithelial cells express CXCR2 (Vanderbilt *et al.*, 2003). Future work to address potential differences of the Reparixin effect on endothelial, epithelial and neutrophil CXCR2 may reveal interesting differences.

The incidence of aspiration-induced ALI is especially high in trauma patients and during and following surgery (Marion, 1991; Hardman and O'Connor, 1999). The acid contained in aspirated gastric contents may cause direct damage to the alveolar-capillary membrane and induce neutrophil recruitment and an increase of the vascular permeability (Marik, 2001). It has been shown that several inflammatory mediators, including platelet-activating factor (Nagase *et al.*, 1999), thromboxane A_2 (Zarbock *et al.*, 2006) and IL-8 (Folkesson *et al.*, 1995) are involved in the pathogenesis of clinically relevant acid-induced ALI models in animals. In addition, neutrophil-platelet interactions are critical for acid-induced ALI in mice (Zarbock *et al.*, 2006; Zarbock and Ley, 2007). Reparixin not only reduced neutrophil recruitment and vascular permeability in this ALI model, but also improved gas exchange, a key functional parameter.

Blocking CXCR2-induced signalling by Reparixin significantly reduced ischaemia-reperfusion injury in several animal models (Bertini *et al.*, 2004; Souza *et al.*, 2004; Cavalieri *et al.*, 2005; Cugini *et al.*, 2005; Garau *et al.*, 2005). Ongoing phase II clinical trials are testing the efficacy of Reparixin in the prevention and treatment of the delayed graft function in kidney transplants and primary graft dysfunction in lung transplants.

Our experimental data are very promising for future clinical applications of CXCR2 inhibitors in ALI. The first phase of aspiration-induced ALI is non-bacterial (Marik, 2001), suggesting that neutrophil recruitment can be blocked without negative effects on host defence. The blockade of CXCR2 needs to be selectively applied, as neutrophils are the first line in host defence against bacterial pathogens, and as such the impairment of neutrophil recruitment can have deleterious effects, like increased mortality in a model of bacteria-induced pneumonia (Moore *et al.*, 2000). Taken together, these findings support the clinical application of Reparixin for the early phase of aspiration-induced ALI.

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Conflict of interest

KL received a research grant from Dompé pha.r.ma.

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