

RESEARCH PAPER

Activation of EP₄ receptors prevents endotoxin-induced neutrophil infiltration into the airways and enhances microvascular barrier function

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BACKGROUND AND PURPOSE

Pulmonary vascular dysfunction is a key event in acute lung injury. We recently demonstrated that PGE₂, via activation of E-prostanoid (EP)₄ receptors, strongly enhances microvascular barrier function *in vitro*. The aim of this study was to investigate the beneficial effects of concomitant EP₄ receptor activation in murine models of acute pulmonary inflammation.

EXPERIMENTAL APPROACH

Pulmonary inflammation in male BALB/c mice was induced by LPS (20 µg per mouse intranasally) or oleic acid (0.15 µL·g⁻¹, *i.v.*). *In-vitro*, endothelial barrier function was determined by measuring electrical impedance.

KEY RESULTS

PGE₂ activation of EP₄ receptors reduced neutrophil infiltration, pulmonary vascular leakage and TNF-α concentration in bronchoalveolar lavage fluid from LPS-induced pulmonary inflammation. Similarly, pulmonary vascular hyperpermeability induced by oleic acid was counteracted by EP₄ receptor activation. In lung function assays, the EP₄ agonist ONO AE1-329 restored the increased resistance and reduced compliance upon methacholine challenge in mice treated with LPS or oleic acid. In agreement with these findings, EP₄ receptor activation increased the *in vitro* vascular barrier function of human and mouse pulmonary microvascular endothelial cells and diminished the barrier disruption induced by LPS. The EP₂ agonist ONO AE1-259 likewise reversed LPS-induced lung dysfunction without enhancing vascular barrier function.

CONCLUSION AND IMPLICATIONS

Our results show that activation of the EP₄ receptor strengthens the microvascular barrier function and thereby ameliorates the pathology of acute lung inflammation, including neutrophil infiltration, vascular oedema formation and airway dysfunction. This suggests a potential benefit for EP₄ agonists in acute pulmonary inflammation.

Abbreviations

ALI, acute lung injury; BAL, bronchoalveolar lavage

Tables of Links

TARGETS
GPCRs
EP ₂ receptor
EP ₄ receptor

LIGANDS	
GW627368X	ONO AE1-259
IL-6	ONO AE1-329
LPS	PF-04418948
Misoprostol	PGE ₂
Oleic acid	TNF- α

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in <http://www.guidetopharmacology.org>, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 (Alexander *et al.*, 2013).

Introduction

Pulmonary vascular dysfunction is a key component of acute lung injury (ALI) and its more severe form, acute respiratory distress syndrome (Ware, 2006; Maniatis *et al.*, 2008). Mortality rates of ALI patients are estimated to be 24–41% (Rubenfeld *et al.*, 2005; Rubenfeld and Herridge, 2007). Notably, increased pulmonary microvascular permeability is associated with adverse outcome and even higher mortality rates (Bull *et al.*, 2010). Consequently, substances promoting or protecting microvascular barrier function would seem to be promising candidates for the therapy of ALI and sepsis (Jacobson and Garcia, 2007; Birukov *et al.*, 2013). The major COX product PGE₂ has been shown to have protective effects in pulmonary inflammatory diseases (Pavord *et al.*, 1993; Vancheri *et al.*, 2004; Aggarwal *et al.*, 2010). PGE₂ acts via four E-type prostanoid receptors (designated as EP₁₋₄), which show cell type-specific expression and their activation stimulates different signalling pathways leading to diverse cellular outcomes. Specifically in the lung, PGE₂ reduces bronchoconstriction and blocks proinflammatory cytokine release from alveolar macrophages via EP₄ receptors and prevents lung fibrosis via EP₂ receptors (Konya *et al.*, 2013a). PGE₂ prevented thrombin-induced permeability of pulmonary artery endothelial cells (Birukova *et al.*, 2007), and recently EP₄ receptors were shown to mediate the endothelial barrier protecting effect of PGE₂: In this study, we identified that the PGE₂-EP₄ signalling axis was more protective microvascular, than of macrovascular permeability in cultures of pulmonary endothelial cells (Konya *et al.*, 2013b). Sphingosine-1 phosphate, a well-characterized protector of the endothelial barrier, attenuated adverse outcomes in murine and canine models of ALI (McVerry and Garcia, 2005). Strikingly, we observed *in vitro* that the microvascular barrier-enhancing effect of EP₄ receptor activation was much more pronounced than that of sphingosine-1 phosphate (Konya *et al.*, 2013b).

These findings suggested a fundamental protective role for the PGE₂-EP₄ axis in ALI via protection of the microvascular permeability barrier.

In the present study, we have assessed the regulatory role of PGE₂ and EP₄ receptors in two mouse models of acute pulmonary inflammation. In LPS-induced pulmonary inflammation, PGE₂ via activation of EP₄ receptors reduced neutrophil infiltration, plasma extravasation and TNF- α release. Furthermore, activation of EP₄ receptors inhibited pulmonary vascular permeability induced by intravenously applied oleic acid. Activation of the EP₄ receptor also restored the altered airway resistance and lung compliance after methacholine challenge in mice with LPS- or oleic acid-induced lung inflammation. *In vitro* experiments using human and murine pulmonary microvascular endothelial cells confirmed the *in vivo* results, as we could show that barrier disruption following LPS was diminished by activation of EP₄ receptors.

Methods

Animals

All animal care and experimental procedures used in this study complied with national and international guidelines and were approved by the Austrian Federal Ministry of Science, Research and Economy (BMWFV GZ:66.010/032-II/10b/2012; GZ:66.010/0173-II/3b/2012; GZ:66.010/0009-WF/V/3b/2015), conform with the Directive 2010/63/EU. Studies are reported in compliance with the ARRIVE guidelines (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010). A total of 392 animals were used in the experiments described here. Male BALB/c mice, 6–8 weeks old (body weight 22–25 g), were obtained from Charles River (Sulzfeld, Germany). Mice were housed in individually ventilated cages (four per cage) under controlled conditions of temperature (set point 21°C), air humidity (set point 50%) and a 12 h light/dark cycle (lights

on at 06:00 h) and habituated to the environment for at least 1 week. Standard chow (altromin 1324 FORTI, Altromin, Lage, Germany) and water were provided *ad libitum*. Procedures were performed as humanely as possible to minimize animal suffering. Sample size calculations were performed with SigmaStat12.5 (Systat Software Inc, San Jose, CA, USA) using values obtained in pilot experiments. Mice were randomly assigned before treatment. The number (*n*) of animals used in the different experiments is given in the respective figure legends.

LPS-induced acute pulmonary inflammation

Mice were slightly anaesthetized with ketamine/xylazine (50 mg/5 mg·kg⁻¹ i.p.) for intranasal applications. In preliminary experiments, we determined the LPS (*Escherichia coli* O55:B5) dose that induced moderate but reproducible neutrophilic inflammation in the lung which was comparable to previously published data (Haegens *et al.*, 2009; Peters *et al.*, 2010).

Experiment I: Mice were treated with vehicle or LPS (20 µg per mouse in PBS) or LPS in combination with PGE₂ (20 µg per mouse; Goncalves de Moraes *et al.*, 1996; Peters *et al.*, 2010) intranasally in a volume of 25 µL to each nares at 09:00 h.

Experiment II: Mice were pretreated with vehicle or the EP₄ receptor antagonist GW627368X (10 mg·kg⁻¹) (Lee *et al.*, 2013) s.c., 30 min before intranasal treatment as described in experiment I.

Experiment III: Mice were pretreated with vehicle or the EP₄ receptor antagonist ONO AE3-208 (10 mg·kg⁻¹) (Kabashima *et al.*, 2002; Xu *et al.*, 2014) s.c., 30 min before intranasal application of compounds as described in experiment I.

Experiment IV: Mice were pretreated with vehicle or the EP₂ antagonist PF-04418948 (10 mg·kg⁻¹) (af Forselles *et al.*, 2011) s.c., 30 min before intranasal treatment as described in experiment I.

Experiment V: Mice were treated intranasally with vehicle, LPS (20 µg per mouse) or LPS in combination with the EP₄ receptor agonist ONO AE1-329 (3–10–30 µg per mouse) intranasally in a volume of 25 µL to each nares at 09:00 h.

Experiment VI: Mice were treated intranasally with vehicle, LPS (20 µg per mouse) or LPS in combination with the EP₂ receptor agonist ONO AE1-259 (3–10–30 µg per mouse) intranasally in a volume of 25 µL to each nares at 09:00 h.

Four hours after intranasal treatment, mice were killed with an overdose of pentobarbital (100 mg·kg⁻¹ i.p.), followed by sampling of blood and bronchoalveolar lavage (BAL) fluid as previously described (Sturm *et al.*, 2008). In additional experiments, mice were pretreated with the stable PGE₂ analogue misoprostol (50 µg per mouse, s.c.) (Zaslona *et al.*, 2014) 2 h before (at 08:00 h) and 10 h after intranasal application of LPS (10 µg per mouse). Mice were killed 24 h after LPS as described earlier and BAL fluid was sampled. This dose of LPS was chosen, because we found, in preliminary experiments, a higher dose (20 µg per mouse) resulted in excessive neutrophil infiltration after 24 h (data not shown). Leukocytes in blood and BAL fluid were analysed by flow cytometry

as described below. TNF-α and IL-6 were determined in the BAL fluid by FlowCytomix multiplex kit (eBioscience; Vienna, Austria).

Leukocyte analysis by flow cytometry

BAL fluid and citrated whole blood were centrifuged at 400× *g* for 7 min at 4°C. Supernatants of BAL fluid were collected, aliquoted and stored at -70°C for further analysis. Plasma was discarded and red blood cells were lysed using BD FACS lysing solution (BD Biosciences, Vienna, Austria). Following two washing steps with Ca²⁺ and Mg²⁺ free PBS, cells were incubated for 30 min at 4°C with the following monoclonal antibodies: FITC-conjugated MHC-II (2.5 µg·mL⁻¹), PE-conjugated CCR3 antibody (2 µg·mL⁻¹), PE-Cy5.5-conjugated CD3e antibody (2 µg·mL⁻¹), PE-Cy7-conjugated B220 antibody (2 µg·mL⁻¹), APC-conjugated CD11c antibody (2 µg·mL⁻¹) and CD16 Block (2 µg·mL⁻¹) (all antibodies were purchased from BD Biosciences). Samples were washed once in PBS, 200 µL fixative solution was added and samples were kept on ice until analysis. Cell suspension was measured for 30 s on a FACSCalibur flow cytometer. Cell populations were analysed as events per 30 s on FlowJo software (Treestar, Costa Mesa, CA, USA; version vX.0.7). Cell populations were identified with adaptations as described elsewhere (van Rijt *et al.*, 2004; Sturm *et al.*, 2008). In detail, lymphocytes were identified as FSC^{low}/SSC^{low} CD3^{high}/B220^{high} cells and further divided into MHC-II^{neg} T- and MHC-II^{high} B-lymphocytes. FSC^{med}, SSC^{med}, CD3^{neg}/B220^{neg} granulocytes were identified as CCR3^{neg} neutrophils and CCR3^{high} eosinophils. FSC^{high}/SSC^{med} alveolar macrophages were gated as CD11c^{high}/MHC-II^{high} and monocytes were identified as CD11c^{med}/MHC-II^{neg} cells.

Pulmonary vascular permeability

Mice were treated intranasally with vehicle or LPS, or LPS in combination with PGE₂ or the EP₄ receptor agonist ONO AE1-329 (20 µg per mouse) at 09:00 h as described earlier. Three hours post treatment, mice were injected with Evans blue (60 mg·kg⁻¹ in saline) in the tail vein and 1 h later mice were killed with pentobarbital (100 mg·kg⁻¹, i.p.) and exsanguinated by cutting the abdominal aorta. The lungs were perfused via the right ventricle with 10 mL of PBS containing 5 mmol·L⁻¹ EDTA. Thereafter, lungs were excised and weighed, then homogenized in PBS (1 mL per 100 µg tissue) and incubated with two volumes of formamide (18 h, 60°C). After centrifugation (5000× *g* for 20 min), the absorbance at 620 nm and 740 nm was measured. The Evans blue content of tissue was calculated and corrected for the presence of heme pigments as described (Wang *et al.*, 2002). In order to induce pulmonary vascular leakage with oleic acid, mice were anaesthetized with ketamine (100 mg·kg⁻¹) and xylazine (10 mg·kg⁻¹), and oleic acid (0.15 µL·g⁻¹) together with Evans blue (60 mg·kg⁻¹) was injected into the tail vein at 09:00 h. Immediately thereafter, vehicle or the EP₄ agonist ONO AE1-329 (20 µg per mouse) was applied intranasally. Body temperature was maintained by placing the mice on a heating pad. Ninety minutes later, mice were killed and their lungs were processed as described above.

Assessment of airway function

Mice received intranasal application of vehicle or LPS (20 µg per mouse), the EP₄ receptor agonist ONO AE1-329 (20 µg per

mouse) or the EP₂ receptor agonist ONO AE1-259 (30 µg per mouse) at 09:00 h as described above. Four hours later, lung function was determined by the flexiVent system (flexiVent, Scireq, Montreal, QC, Canada) as described by Nemmar *et al.*, (2011). Briefly mice were anaesthetized with ketamine (150 mg·kg⁻¹) and xylazine (10 mg·kg⁻¹) by i.p. injection. After surgical anaesthesia was established, tracheostomy was performed and an 18 gauge cannula was inserted into the trachea. Animals were placed on a 37°C heating pad, connected to a computer controlled small-animal ventilator and ventilated with a tidal volume of 10 mL·kg⁻¹ at a frequency of 150 breaths per minute and a positive end-expiratory pressure of 2 cm H₂O. Airway resistance (R) and compliance (C) were measured before any treatment (baseline levels) and after delivery of increasing concentrations of nebulized methacholine (1, 3, 10, 30 and 100 mg·mL⁻¹). R and C were defined as the mean out of 12 values for each perturbation if the coefficient of determination was greater than 0.95. Assessment of lung function in mice treated with oleic acid was performed 90 min after injection of oleic acid.

Culture of endothelial cells and endothelial electrical resistance measurements

Human lung microvascular endothelial cells were purchased from Lonza (Verviers, Belgium) and maintained in EGM-2MVBulletKit medium (Lonza) with 5% FCS. Endothelial cells were cultivated as previously described (Konya *et al.*, 2010; El-Gamal *et al.*, 2012). Lung microvascular endothelial cells from BALB/c mice were purchased from Cell Biologics (Chicago, IL, USA) in passage 3 and were cultured until passage 9 in complete Mouse Endothelial Cell Medium Kit (Cell Biologics). Endothelial cells were seeded on 1% gelatine-coated biochips containing gold microelectrodes (ECIS 8W10E+). On the second day after seeding, cells were serum-starved for 1 h and electrical resistance was measured at 4000 Hz frequency using electric cell-substrate impedance sensing system (ECIS, Applied Biophysics, Troy, NY, USA) as described (Konya *et al.*, 2013b). Experiments were performed in duplicate and repeated four to seven times (*n*) with freshly seeded cells per treatment group.

Culture of murine alveolar macrophages

Mice were killed with pentobarbital (100 mg·kg⁻¹, i.p.) at 08:00 h and exsanguinated by cutting the abdominal aorta. BAL was performed with 12 mL PBS containing 1 mmol·L⁻¹ EDTA and 10 mmol·L⁻¹ HEPES, pH 7.3. BAL from three mice was pooled and centrifuged at 400×*g* for 7 min. Erythrocytes were lysed by adding ice-cold ammonium chloride. The washed cell pellet was resuspended in 1 mL complete RPMI medium containing 10% FCS, 1% penicillin/streptomycin and cells were counted. Cells (4 × 10⁴ per well) were seeded in 100 µL of medium in 96-well plates. Macrophages were allowed to adhere for 1 h at 37°C in a 5% CO₂ humidified incubator. Non-adherent cells and debris were removed by washing (Peters *et al.*, 2010). The adherent macrophages were cultured in 100 µL of fresh complete medium and stimulated with LPS (10 ng·mL⁻¹) in the absence or presence of PGE₂ (30–300 nmol·L⁻¹) or the specific EP₄ agonist ONO AE1-329 (30–300 nmol·L⁻¹). In a subset of experiments, cells were pre-treated with the EP₄ antagonists ONO AE3-208 (100 nmol·L⁻¹)

or GW627368X (100 nmol·L⁻¹) for 30 min before being exposed to LPS and PGs. Cells were treated for 4 h, and thereafter the supernatant was collected for determination of TNF-α and IL-6 using FlowCytomix kit (eBioscience). These experiments were performed in triplicate and repeated five to eight times (*n*) with cells from different donors per group.

Lung histology and EP₄ receptor immunohistochemistry

Mice were killed with pentobarbital (100 mg·kg⁻¹) at 08:00 h, their lungs were perfused via the pulmonary artery with 0.1 mol·L⁻¹ PBS (pH 7.4) followed by 4% buffered paraformaldehyde (Sturm *et al.*, 2008). The lungs were dissected, post-fixed for 24 h and embedded in paraffin. Five micrometre sections were deparaffinized and either haematoxylin/eosin staining was performed, or sections were immunostained for EP₄ receptors.

For EP₄ receptor staining, antigen retrieval was performed with antigen unmasking solution pH 6.0 (Vector, Vector Labs, Burlingame, CA, USA) and to reduce non-specific background, sections were incubated in blocking solution (Vectastain ABC Kit, Vector Labs). Thereafter, samples were incubated with a polyclonal EP₄ antibody (AB133170; 1:400 diluted; Abcam, Cambridge, UK) overnight. Samples were further incubated for 45 min with a goat anti-rabbit antibody (Jackson Immunoresearch, West Grove, PA, USA; 1:200 diluted). After washing, the bound antibody was detected using the Vectastain ABC kit and 3-amino-9-ethylcarbazole as chromogen and counterstained with haematoxylin. Sections were visually examined with an Olympus BX41 microscope (Olympus Austria GmbH, Vienna, Austria) and an Olympus U Plan Apo ×40/0.8 or ×60/0.8 lens. Photographs were taken with an Olympus DP50 camera (2776 × 2074 pixels) and further processed with CELL^P software (Olympus Austria GmbH) for additional white balance, contrast and brightness adjustments.

Data analysis

Statistical analysis was performed using GraphPad Prism software 6.0 (La Jolla, CA, USA). Experiments using endothelial cells and alveolar macrophages were performed in duplicate or triplicate, respectively, and were repeated *n* times with freshly seeded cells or with cells from different donors. Concentration–response curves were compared with each other using one-way ANOVA for repeated measurements; the differentially treated groups of mice were tested for significant differences with one-way ANOVA; lung function and electrical resistance measurements were analysed by two-way ANOVA. Statistical differences between groups were determined by Tukey's multiple comparison test. Probability values of *P* < 0.05 were regarded as statistically significant.

Materials

All laboratory reagents were from Sigma (Vienna, Austria) unless specified. The EP₄ antagonist GW627368X was obtained from Cayman (Tallin, Estonia). ONO AE1-329, ONO AE1-259 and ONO AE3-208 were generous gifts from ONO Pharmaceutical (Osaka, Japan). CellFix and FACS-Flow from BD Bioscience. Fixative solution was prepared by adding 9 mL distilled water and 30 mL FACS-Flow to 1 mL CellFix.

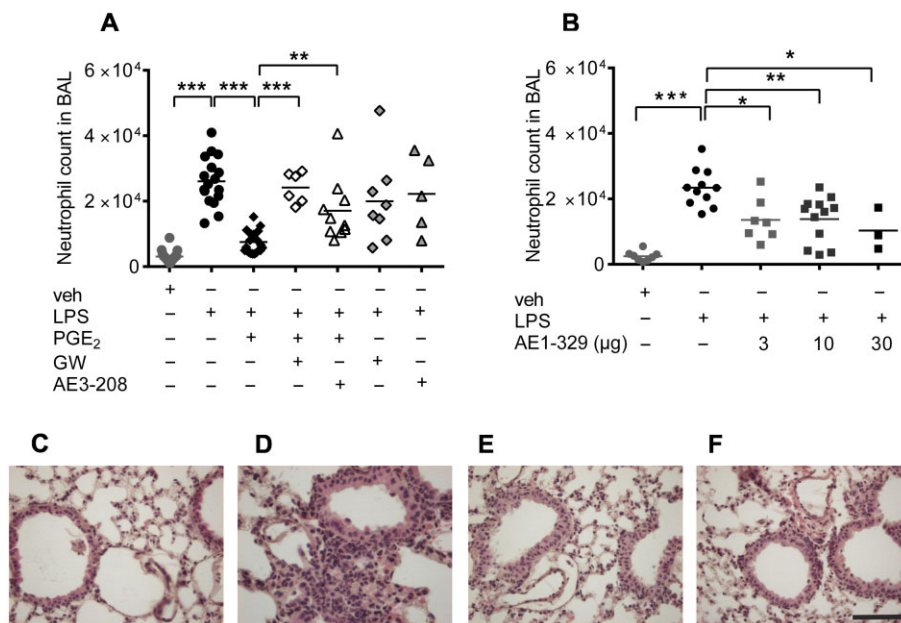


Figure 1

Neutrophil counts in BAL fluid of (A) mice treated intranasally with vehicle (veh; $n = 18$), LPS (20 μg per mouse; $n = 17$), PGE₂ (20 μg per mouse) concomitantly with LPS ($n = 22$) or pretreated with the EP₄ receptor antagonists GW627368X (GW; 10 mg·kg⁻¹ s.c.; $n = 8$) or ONO AE3-208 (10 mg·kg⁻¹ s.c.; $n = 5$) 30 min before intranasal LPS or LPS concomitantly applied with PGE₂ ($n = 6$ or 10 respectively). (B) Intranasal application of vehicle (veh, $n = 8$) or LPS (20 μg per mouse, $n = 11$) alone or in combination with the EP₄ receptor agonist ONO AE1-329 ($n = 3$ –10). Each point shows the neutrophil count in BAL fluid from one animal. Data were analysed using one-way ANOVA and multiple comparisons were calculated with Tukey's post test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. (C–F) Haematoxylin/eosin staining of lungs with intranasal application of (C) vehicle, (D) LPS (20 μg per mouse), (E) PGE₂ (20 μg per mouse) given concomitantly with LPS and (F) ONO AE1-329 (20 μg per mouse) concomitantly applied with LPS. Lung histology micrographs are representative of three independent experiments; scale bar = 50 μm.

PGE₂, ONO AE1-329 and ONO AE1-259 were dissolved in ethanol, while stock solutions of GW627368X, PF-04418948, ONO AE3-208 and misoprostol were prepared in DMSO and further diluted in PBS to produce a final concentration of the solvents of less than 0.1%.

Results

PGE₂ via EP₄ receptor activation inhibits LPS-induced neutrophil infiltration in the lung

Intranasal treatment of mice with LPS (20 μg per mouse) caused a pronounced increase in neutrophil counts in the BAL fluid (Figure 1A) after 4 h, with only a moderate increase in monocyte and T-lymphocyte count (see Supporting Information Fig. S1). In parallel, an increase of neutrophils was also observed in blood samples (see Supporting Information Fig. S2). In additional experiments, treatment with the stable PGE₂ analogue misoprostol (50 μg per mouse), which was used here because of the short half-life of PGE₂, abolished neutrophilia in the BAL fluid 24 h after intranasal application of LPS (10 μg per mouse; see Supporting Information Fig. S3). All further experiments were performed 4 h after LPS challenge. The LPS-induced neutrophilia in the airways was abolished by co-administration with PGE₂ (20 μg per mouse)

(Figure 1A). Notably, pretreatment of the animals with the EP₄ receptor antagonist GW627368X or ONO AE3-208 (both at 10 mg·kg⁻¹ s.c.), 30 min before LPS/PGE₂ co-administration reversed the inhibitory effect of PGE₂ on LPS-induced pulmonary neutrophil infiltration, but had no effect on neutrophil counts in the absence of PGE₂ (Figure 1A). To further substantiate this finding, LPS (20 μg per mouse) was intranasally applied in combination with the selective EP₄ receptor agonist ONO AE1-329 (3–30 μg per mouse), which likewise caused a reduction of BAL neutrophils (Figure 1B). These observations were reflected at the tissue level where the dense leukocyte infiltration seen after LPS (Figure 1D) treatment was markedly reduced by PGE₂ and ONO AE1-329 (Figure 1E and F respectively). In addition, we found that pretreatment of mice with the EP₂ receptor antagonist PF-04418947 (10 mg·kg⁻¹ s.c.) counteracted the effect of PGE₂ while intranasal co-administration with the EP₂ receptor agonist ONO AE1-259 (3–30 μg per mouse) exerted only minor effects on LPS-induced neutrophil infiltration (see Supporting Information Fig. S4).

PGE₂ via EP₄ receptor activation inhibits LPS-induced pulmonary vascular leakage

Intranasal application of LPS (20 μg per mouse) caused significant vascular leakage after 4 h, which could be inhibited by co-administration of PGE₂ (20 μg per mouse) (Figure 2A),

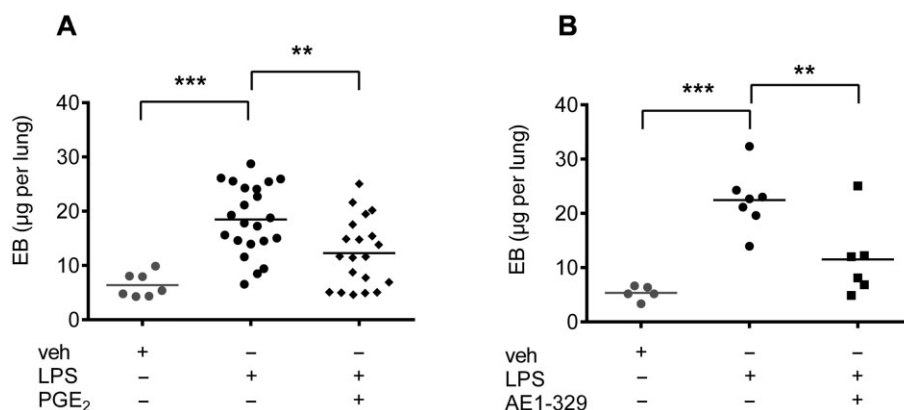


Figure 2

Evans blue extravasation in the lung of mice intranasally treated (A) with vehicle (veh, $n = 7$), LPS (20 μg per mouse, $n = 22$) or LPS in combination with PGE₂ (20 μg per mouse, $n = 20$). (B) Intranasal treatment with vehicle (veh, $n = 5$), LPS (20 μg per mouse, $n = 7$) or in combination with the EP₄ receptor agonist ONO AE1-329 (20 μg per mouse, $n = 6$). Each point shows μg Evans blue (EB) per lung from one animal. Data were analysed using one-way ANOVA and multiple comparisons were calculated with Tukey's post test. ** $P < 0.01$, *** $P < 0.001$.

as well as by the EP₄ receptor agonist ONO AE1-329 (20 μg per mouse) (Figure 2B). These data suggest that LPS-induced pulmonary vascular leakage is inhibited by PGE₂ via EP₄ receptor activation.

Effect of PGE₂ and EP₄ receptor activation on LPS-induced cytokine release

Cytokine concentrations were determined in BAL fluid which had been obtained from the experiments described earlier. Low levels of IL-1 α , IL-2, IL-5, IL-10 and GM-CSF were found while IFN- γ and IL-17 were undetectable in the BAL fluid (data not shown). LPS (20 μg per mouse) caused an increase of TNF- α and IL-6 that was significantly inhibited by PGE₂ (20 μg per mouse; Figure 3A and B). Unexpectedly, this inhibitory effect of PGE₂ on cytokine release was not influenced by pretreatment of mice with the EP₄ receptor antagonists GW627368X (10 $\text{mg}\cdot\text{kg}^{-1}$ s.c.) or ONO AE3-208 (10 $\text{mg}\cdot\text{kg}^{-1}$ s.c.; Figure 3A and B), or by the EP₂ receptor antagonist PF-04418948 (10 $\text{mg}\cdot\text{kg}^{-1}$ s.c.; Supporting Information Fig. S5). Interestingly, concomitant application of the specific EP₄ receptor agonist ONO AE1-329 or the EP₂ receptor agonist ONO AE1-259 (at a dose of 30 μg per mouse) along with LPS abolished the increase of TNF- α , without affecting the increase in IL-6 (Figure 3C and D, and Supporting Information Fig. S5).

Because TNF- α is derived predominantly from activated macrophages, we isolated bronchoalveolar macrophages from mice. In the supernatants of unstimulated macrophages, we were unable to detect any TNF- α ; however, treatment with LPS (10 $\text{ng}\cdot\text{mL}^{-1}$) for 4 h caused an increase in TNF- α that could be significantly inhibited by PGE₂ (100–300 $\text{nmol}\cdot\text{L}^{-1}$; Figure 4A). Pretreatment of the cells with the EP₄ receptor antagonists ONO AE3-208 (300 $\text{nmol}\cdot\text{L}^{-1}$) or GW627368X (1 $\mu\text{mol}\cdot\text{L}^{-1}$) blocked the inhibitory effect of PGE₂ (30 $\text{nmol}\cdot\text{L}^{-1}$) on TNF- α release (Figure 4B). In addition, the EP₄ receptor agonist ONO AE1-329 mimicked the effect of PGE₂ (Figure 4C). Interestingly, both PGE₂ (30 $\text{nmol}\cdot\text{L}^{-1}$) and ONO AE1-329 (100 $\text{nmol}\cdot\text{L}^{-1}$) augmented the secretion of IL-6

from alveolar macrophages (Figure 4D), and this was inhibited by ONO AE3-208 (300 $\text{nmol}\cdot\text{L}^{-1}$), which contrasted with the reduction by PGE₂ of the IL-6 content of lungs *in vivo* (Figure 3B). These observations suggested that EP₄ receptor activation might be involved in the regulation of TNF- α secretion, while additional PG receptors, and possibly cells other than macrophages, were likely to mediate the effect of PGE₂ on cytokine release, in particular IL-6.

Activation of EP₄ receptors promotes pulmonary microvascular endothelial barrier function in vitro

In cultures of murine lung endothelial cells, PGE₂ (3–300 $\text{nmol}\cdot\text{L}^{-1}$) and the selective EP₄ receptor agonist ONO AE1-329 (0.1–30 $\text{nmol}\cdot\text{L}^{-1}$) (Figure 5A and B) increased endothelial barrier function, and ONO AE3-208 prevented the endothelial barrier enhancement induced by the EP₄ receptor agonist (30 $\text{nmol}\cdot\text{L}^{-1}$) and PGE₂ (30 $\text{nmol}\cdot\text{L}^{-1}$) (Figure 5C and D). In agreement with these functional observations, flow cytometric analysis confirmed that murine pulmonary microvascular endothelial cells in culture expressed EP₄ receptors (see Figure 5E). These observations extend our previous findings made in human endothelial cells (Konya *et al.*, 2013b). In contrast, the EP₂ receptor agonist ONO AE1-259 (up to 100 $\text{nmol}\cdot\text{L}^{-1}$) did not affect the barrier function (data not shown; $n = 3$).

We have recently reported that activation of EP₄ receptors on human endothelial cells strengthens the barrier function of the monolayer (Konya *et al.*, 2013b). Here, we showed that LPS (1 $\mu\text{g}\cdot\text{mL}^{-1}$) 1.6 h after addition, decreased endothelial barrier function *in vitro*, as demonstrated by reduced electrical resistance. The EP₄ receptor agonist ONO AE1-329 (100 $\text{nmol}\cdot\text{L}^{-1}$) or PGE₂ (30 $\text{nmol}\cdot\text{L}^{-1}$) in the presence of LPS caused an increase in barrier function for up to 2.1 and 2.9 h, respectively, and attenuated the LPS effect for up to 7 h (Figure 6A and B). Pre-incubation with the EP₄ receptor-selective antagonist ONO AE3-208 (300 $\text{nmol}\cdot\text{L}^{-1}$) abolished

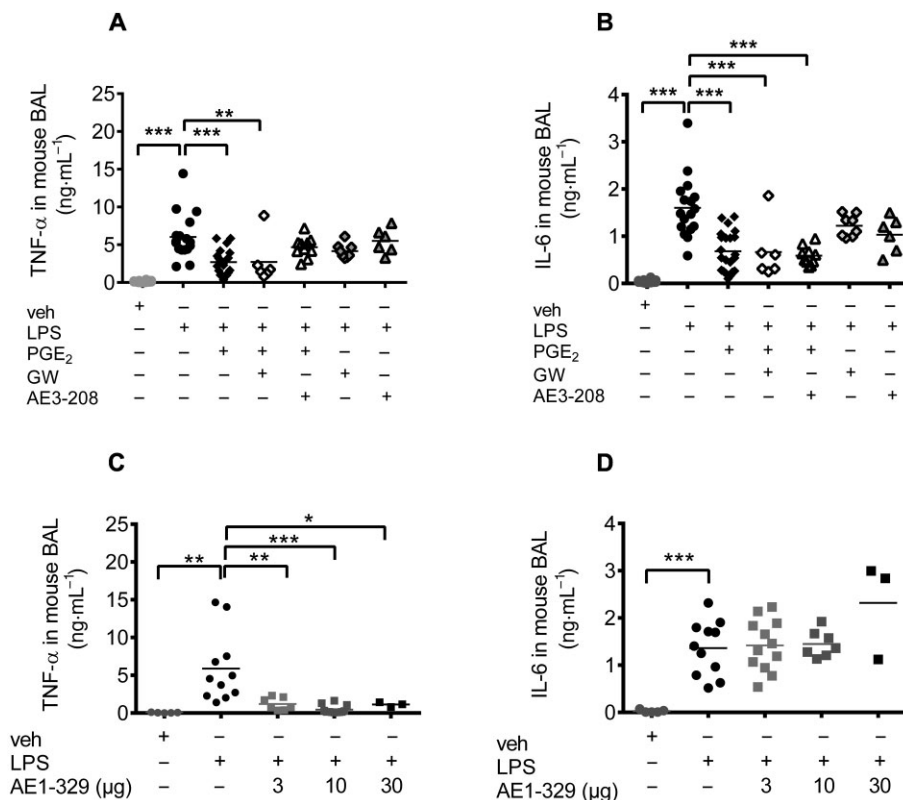


Figure 3

TNF- α (A,C) and IL-6 (B,D) levels in the BAL fluid of (A,B) mice treated intranasally with vehicle (veh; $n = 18$), LPS (20 μ g per mouse; $n = 17$), PGE₂ (20 μ g per mouse) concomitantly with LPS ($n = 22$) or pretreated with the EP₄ receptor antagonists GW627368X (GW; 10 mg·kg⁻¹ s.c.; $n = 8$) or ONO AE3-208 (10 mg·kg⁻¹ s.c.; $n = 5$) 30 min before intranasal LPS or LPS concomitantly applied with PGE₂ ($n = 6$ or 10 respectively). (C,D) Intranasal application of vehicle (veh, $n = 8$) or LPS (20 μ g per mouse, $n = 11$) alone or in combination with the EP₄ receptor agonist ONO AE1-329 ($n = 3$ –10). Each point shows the cytokine concentration (ng mL⁻¹) from one animal. Data were analysed using one-way ANOVA and multiple comparisons were calculated with Tukey's post test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

the endothelial barrier enhancement induced by the EP₄ receptor agonist and PGE₂ (30 nmol·L⁻¹) in the presence of LPS (Figure 6C and D). This was the first time that EP₄ receptor activation had been shown to prevent the barrier disruption induced by LPS.

Moreover, immunohistochemistry of murine lung tissue demonstrated pulmonary endothelial cells to stain positively for EP₄ receptors (Figure 7) while no signal was detected with isotype control (see Supporting Information Fig. S6).

EP₄ receptor activation attenuates oleic acid-induced vascular leakage in the lung

These observations indicated that stimulation of EP₄ receptors on endothelial cells might be involved in protection against acute pulmonary inflammation. To further substantiate this notion, we used another pulmonary inflammation model that is primarily based on pulmonary vascular injury and investigated the involvement of EP₄ receptors in oleic acid-induced vascular leakage. Intravenous administration of oleic acid (0.15 μ L·g⁻¹ body weight) caused a pronounced increase in Evans blue extravasation in the lung after 90 min. Intranasal treatment of the mice with the selective EP₄ recep-

tor agonist ONO AE1-329 (20 μ g) significantly diminished this response (Figure 8), arguing for an important role of EP₄ receptors in endothelial protection.

EP₄ receptor activation attenuates LPS- and oleic acid-induced airway hyperresponsiveness to methacholine

Finally, we investigated whether EP₄ receptor-mediated lung protection also translates to regulation of respiratory function. Four hours after intranasal application of LPS (20 μ g per mouse) and 90 min after i.v. injection of oleic acid (0.15 μ L·g⁻¹ body weight), no changes in baseline resistance and compliance were seen in mechanically ventilated mice; however, significantly increased responses to aerosolized methacholine (10–100 ng·mL⁻¹) were observed with respect to resistance and compliance as compared with vehicle-treated control mice (Figure 9). While concomitant treatment with PGE₂ did not significantly affect resistance, it showed a minor improvement of compliance. The EP₄ receptor agonist ONO AE1-329 (20 μ g per mouse) reversed the airway hyperresponsiveness induced by LPS and by oleic acid (Figure 9). As it is known that the EP₂ receptor mediates the bronchodilator

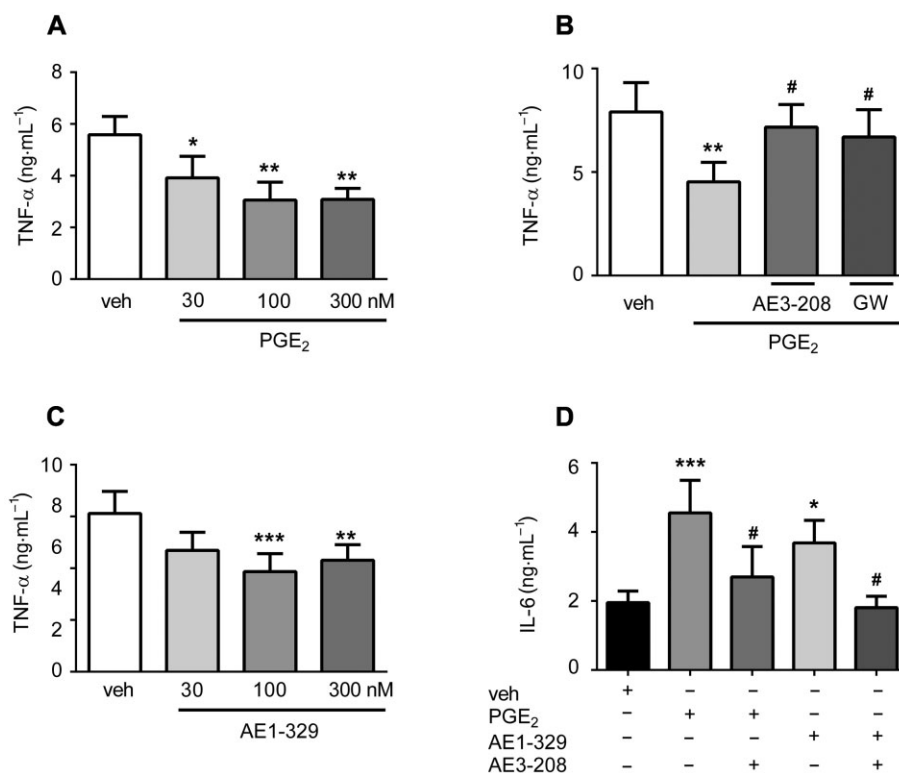


Figure 4

TNF- α (A–C) and IL-6 (D) concentrations in the supernatants of isolated murine alveolar macrophages. Cells were treated with LPS (10 ng·mL⁻¹) for 4 h in the presence of (A) vehicle (veh) or PGE₂ (concentrations as indicated), $n = 5$, (B) vehicle (veh) or PGE₂ (30 nmol·L⁻¹) in the absence or presence of the EP₄ receptor antagonists ONO AE3-208 (300 nmol·L⁻¹) or GW627368X (GW, 1 μ mol·L⁻¹) for 30 min, $n = 6$, (C) vehicle (veh) or the EP₄ receptor agonist ONO AE1-329 (concentrations as indicated), $n = 8$, (D) vehicle (veh) or PGE₂ (30 nmol·L⁻¹) or ONO AE1-329 (100 nmol·L⁻¹) in the absence or presence of the EP₄ receptor antagonist ONO AE3-208 (300 nmol·L⁻¹), $n = 7$. Data are shown as mean + SEM of n independent experiments performed in triplicate. Data were analysed using one-way ANOVA for repeated measurements and multiple comparisons were calculated with Tukey's post test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ as compared with LPS treatment, # $P < 0.05$ as compared with PGE₂ or EP₄ agonist treatment.

effect of PGE₂ in mice, we investigated this in our model. The EP₂ receptor agonist ONO AE1-259 (30 μ g per mouse) given concomitantly with LPS intranasally, also significantly inhibited the LPS-induced increase in resistance and decrease in compliance (see Supporting Information Fig. S7).

Discussion

In this study, we have demonstrated that concomitant activation of the PGE₂ receptor, EP₄, ameliorates LPS-induced lung pathology, including recruitment of neutrophils, plasma extravasation and airway dysfunction, which are key clinical features of ALI (Abraham, 2003). We provide evidence that one major mechanism behind these beneficial PGE₂ effects is via facilitation of endothelial barrier function through activation of endothelial EP₄ receptors. A second mechanism appears to be the reduction of cytokine secretion observed after PGE₂ administration. The limiting role of PGE₂ in LPS-induced neutrophil accumulation has been recognized previously (Goncalves de Moraes *et al.*, 1996; Peters *et al.*, 2010).

Our data corroborate these findings. The crucial finding of our study, however, is that activation of EP₄ receptors is largely responsible for this, as the protective PGE₂ effect was prevented by the EP₄ receptor antagonists GW627368X and ONO AE3-208, and was mimicked by the selective EP₄ receptor agonist ONO AE1-329. In addition, we also found an involvement of the EP₂ receptor, as the EP₂ receptor antagonist PF-04418948 blocked the inhibitory effect of PGE₂ on neutrophil accumulation. However, the inhibitory effect of the highly selective EP₂ receptor agonist ONO AE1-259 (Maruyama and Ohuchida, 2000) did not reach statistical significance. The EP₄ and EP₂ receptor antagonists by themselves did not change neutrophil infiltration induced by LPS. This was unexpected, as LPS is known to increase COX-2 expression and increase PG synthesis (Lin *et al.*, 1996; Amann *et al.*, 1999; Murakami *et al.*, 2000). Nevertheless, the endogenous levels of PGE₂ may be too low in our experimental model to affect neutrophil infiltration. Accordingly, we observed only a minor (twofold) increase in the levels of PGE₂ metabolites in the BAL fluid after LPS treatment (data not shown). In a similar model, however, Birrell *et al.* (2015) found that EP₄ receptor knockout mice showed exaggerated

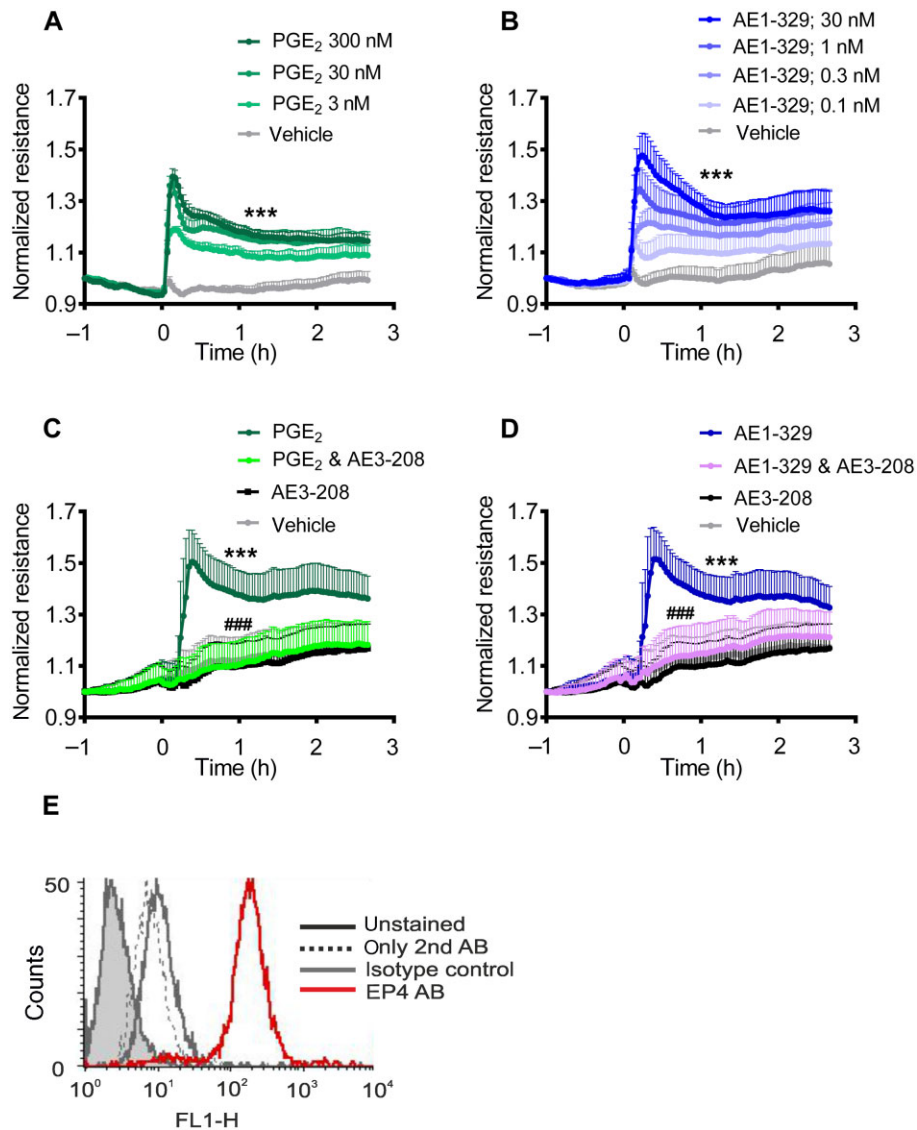


Figure 5

Electrical resistance of murine endothelial cell monolayers treated with either vehicle or (A) PGE₂ or the (B) EP₄ agonist ONO AE1-329 at the indicated concentrations. (C). Cells were treated with PGE₂ (30 nmol·L⁻¹) or (D) the EP₄ agonist ONO AE1-329 (30 nmol·L⁻¹) alone or in the presence of the EP₄ antagonist ONO AE3-208 (1 μmol·L⁻¹, 15 min pretreatment). Data are shown as mean normalized resistance + SEM of four independent experiments performed in duplicate. Data were analysed by two-way ANOVA for repeated measurements and multiple comparisons were calculated with Tukey's post test. ****P* < 0.001 as compared with vehicle; ###*P* < 0.001 as compared with PGE₂ or the EP₄ agonist. (E) EP₄ receptor expression on murine pulmonary microvascular cells using flow cytometry (typical example of *n* = 3). AB, antibody.

responses to LPS and other inflammatory stimuli in the lung. These data were published while our current study was under revision.

Endothelial barrier dysfunction in the lung leads to increased vascular permeability to fluid and macromolecules, resulting in pulmonary oedema. In the present study, we obtained evidence in two *in vivo* models, which are LPS and oleic acid-induced pulmonary vascular dysfunction, that PGE₂ and an EP₄ receptor-selective analogue were capable of strengthening the endothelial barrier, thereby preventing increased vascular permeability in the lungs.

These results are compatible with our *in vitro* data obtained recently with human microvascular endothelial cells, in which neutrophil adhesion to, and migration through, endothelial monolayers *in vitro* was blocked by EP₄ receptor activation, which we could link to reduced E-selectin expression and enhanced barrier function respectively (Konya *et al.*, 2013b). The latter effect was reflected by enhanced electrical resistance and increased formation of adherens junctions by vascular endothelial-cadherin. In extension of this idea, we have demonstrated in the current study that the LPS-induced disruption of endothelial barrier

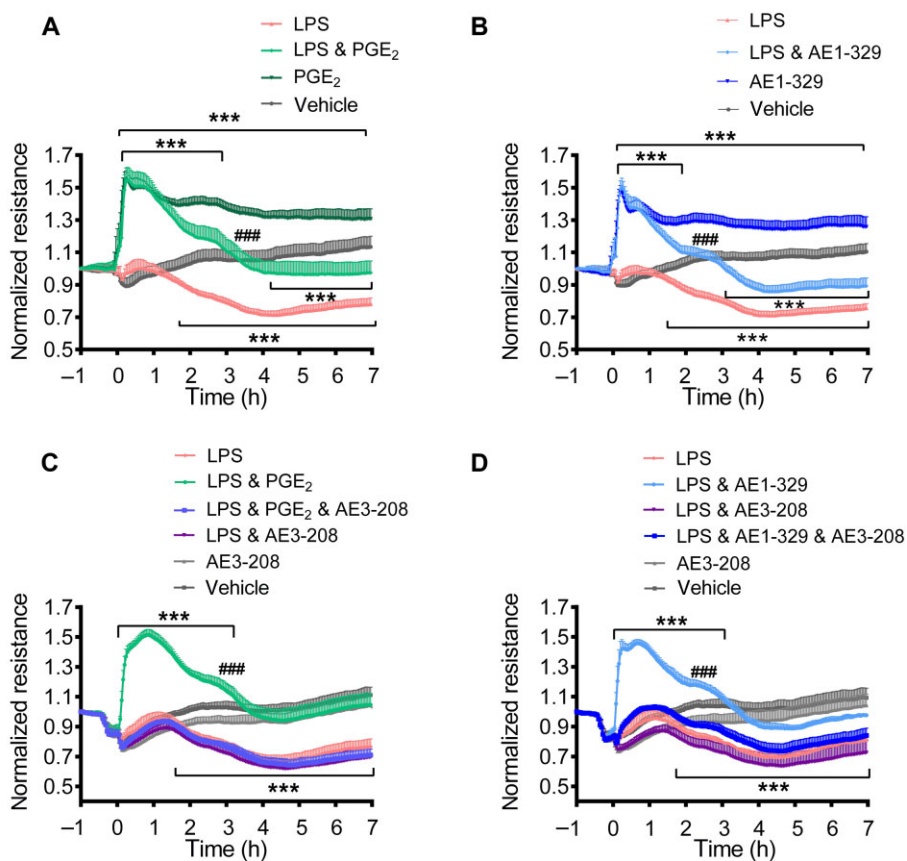


Figure 6

Electrical resistance of human endothelial cell monolayers treated with vehicle or LPS ($1 \mu\text{g}\cdot\text{mL}^{-1}$) in the absence or presence of (A,C) PGE_2 ($30 \text{ nmol}\cdot\text{L}^{-1}$), (B,D) the EP_4 receptor agonist ONO AE1-329 ($100 \text{ nmol}\cdot\text{L}^{-1}$). (C,D) Cells were pretreated with the EP_4 receptor antagonist ONO AE3-208 ($300 \text{ nmol}\cdot\text{L}^{-1}$) for 15 min. Data are shown as mean normalized resistance + SEM of 4–7 independent experiments performed in duplicate. Data were analysed by two-way ANOVA for repeated measurements and multiple comparisons were calculated with Tukey's post test. $***P < 0.001$ as compared with vehicle; $###P < 0.001$ as compared with LPS.

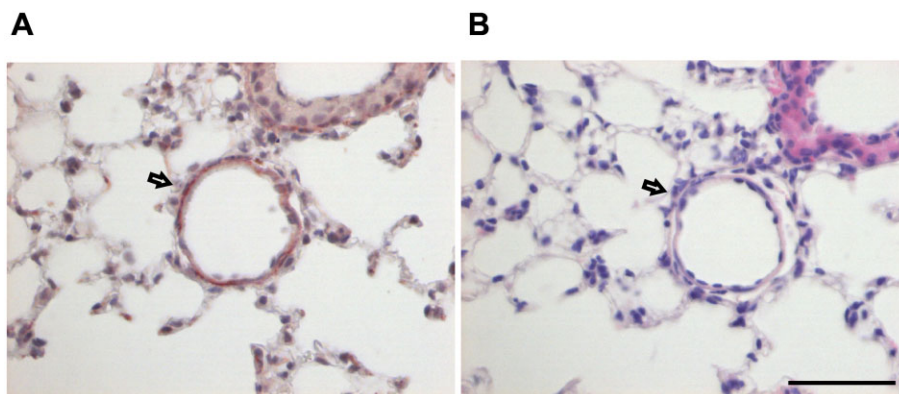


Figure 7

Immunohistochemical staining of EP_4 receptors in murine lung tissue. (A) The endothelial layer of a lung vessel as indicated by the arrow is stained brown with the EP_4 receptor antibody. (B) Haematoxylin–eosin staining of a consecutive section which shows the same vessel. Scale bar = $50 \mu\text{m}$. Micrographs are representative for five lungs.

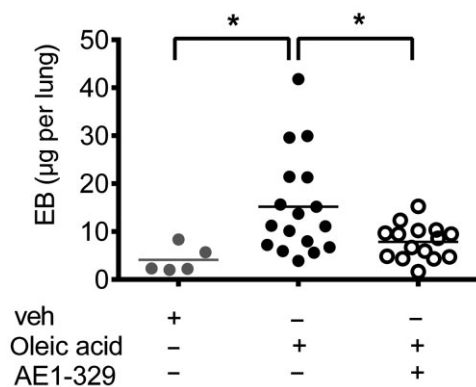


Figure 8

Evans blue (EB) extravasation in the lung of mice that received vehicle (veh, $n = 5$) or oleic acid ($0.15 \mu\text{L}\cdot\text{g}^{-1}$, $n = 17$) injected into the tail vein. The EP₄ receptor agonist ONO AE1-329 ($20 \mu\text{g}$ per mouse, $n = 15$) was given intranasally. Each point shows microgram EB per lung of one animal 90 min after oleic acid injection. Data were analysed by one-way ANOVA and multiple comparisons were calculated with Tukey's post test. * $P < 0.05$.

function in human pulmonary microvascular endothelial cells was prevented by PGE₂ via EP₄ receptor activation.

As already observed with human microvascular lung endothelial cells (Konya *et al.*, 2013b), our present data demonstrate that PGE₂ was likewise able to increase basal vascular barrier function in mouse pulmonary microvascular endothelial cells *in vitro*, reflected by an increase in electrical resistance. This effect was mimicked by the EP₄ receptor agonist and was counteracted by the EP₄ receptor antagonist. Our immunohistochemical data also showed that the EP₄ receptor was expressed on endothelial layers of lung parenchymal vessels in the mouse lung. In contrast, we did not find the EP₂ receptor to be involved in barrier function.

Recent studies have shown that the stable PGI₂ analogue iloprost increases endothelial barrier function, which is mediated by PKA/Epac/Rap1/Rac, and leads to improvement of ventilation-induced ALI as well as LPS-induced ALI, with regard to neutrophil infiltration, increased protein content in BAL fluid and Evans blue extravasation (Birukova *et al.*, 2010; 2013). *In vitro*, iloprost treatment suppressed the LPS-induced cytoskeletal alterations and inflammatory signalling in endothelial cells (Birukova *et al.*, 2013). In addition, Peters *et al.* (2010) reported on the role of the EP₂ receptor in LPS-induced ALI, as butaprost mimicked the effect of PGE₂ in inhibiting neutrophil accumulation in BAL fluid. Here, we confirmed the effect of EP₂ receptor activation on neutrophil infiltration in our model, but this latter effect seems to be unrelated to a direct effect on the endothelial barrier.

In addition to increased vascular permeability and influx of neutrophils into the lung, ALI is also characterized by increased levels of proinflammatory cytokines (Abraham, 2003; Lesur *et al.*, 2010). TNF- α and IL-6 are elevated in ALI and cause vascular barrier dysfunction, oedema formation as

well as orchestrating neutrophil recruitment into lung tissue (Horvath *et al.*, 1988; Millar *et al.*, 1989; Hocking *et al.*, 1990; Birukova *et al.*, 2012). However, whether IL-6 is actually a proinflammatory or anti-inflammatory cytokine in ALI is not clear as, in LPS-induced lung injury, IL-6 plays an anti-inflammatory role (Xing *et al.*, 1998; Bhargava *et al.*, 2013), while in a 'two hit' murine model of LPS and ventilator-induced lung injury it showed an overall proinflammatory effect (Goldman *et al.*, 2014). We found increased TNF- α and IL-6 in the BAL fluid of mice which had been treated intranasally with LPS. As expected, exogenous PGE₂ prevented the increase in TNF- α . Treatment of mice with the EP₄ receptor antagonists GW627368X and ONO AE3-208 as well as the EP₂ receptor antagonist PF-04418948 did not prevent the inhibitory effect of PGE₂. However, intranasal administration of the EP₄ receptor agonist ONO AE1-329 as well as the EP₂ receptor agonist ONO AE1-259, albeit at a higher dose, mimicked the effect of PGE₂. Therefore, the inhibitory effect of PGE₂ on TNF- α *in vivo* seems to be mediated by both receptors and this could explain why blockade of either receptor alone was not effective.

By sharp contrast to TNF- α release, PGE₂ and EP₄ receptor activation were found to increase IL-6 production in macrophages as well as in neutrophils, smooth muscle cells and fibroblasts (Yokoyama *et al.*, 2013). While we corroborated this observation in isolated alveolar macrophages, we also found that exogenous PGE₂ treatment of mice attenuated the LPS-induced increase of IL-6 in BAL fluid. Even more strikingly, EP₄ or EP₂ receptor blockade did not reverse the inhibition of IL-6 secretion by PGE₂, just as EP₄ and EP₂ agonists did not influence IL-6 secretion *in vivo*, while reversing the inhibitory effect of PGE₂ on neutrophil recruitment. Interestingly, a recent study showed that PGE₂ inhibits IL-6 release in mouse and human monocytic cell lines (Birrell *et al.*, 2015). In the lung, in addition to macrophages, a variety of cells produce IL-6, such as epithelial cells, endothelial cells, interstitial fibroblasts and other inflammatory cells (Rincon and Irvin, 2012). Therefore, we assume that the inhibition of IL-6 release by PGE₂ *in vivo* involves other cell types than macrophages. Due to the lack of effect of the EP₄ and EP₂ agonists and antagonists *in vivo*, it remains to be established which EP receptor (EP₁ or EP₃ or IP) and which cell type is involved in the PGE₂ regulation of IL-6 secretion in the lungs.

Proinflammatory cytokines in the lungs can lead to airway hyperresponsiveness (Brightling *et al.*, 2008; Rincon and Irvin, 2012) which we measured in ventilated mice by invasive spirometry. LPS- and oleic acid-induced pulmonary inflammation was accompanied by enhanced bronchoconstrictor responses and decreased compliance after methacholine inhalation. Importantly, treatment of mice with the EP₄ receptor agonist completely normalized LPS- and oleic acid-induced airway dysfunction to levels observed in vehicle-treated mice. In this respect, the EP₄ receptor agonist had a greater effect than PGE₂ at the given dose, as PGE₂ largely corrected the enhanced fall in compliance elicited by methacholine, but had little effect on bronchoconstrictor responses. This discrepancy might be explained by the fact that PGE₂ acts on three additional receptors, that is EP₁₋₃, of which EP₁ and EP₃ receptors have been described as mediating bronchoconstriction in mice, by activating cholinergic reflexes (Tilley

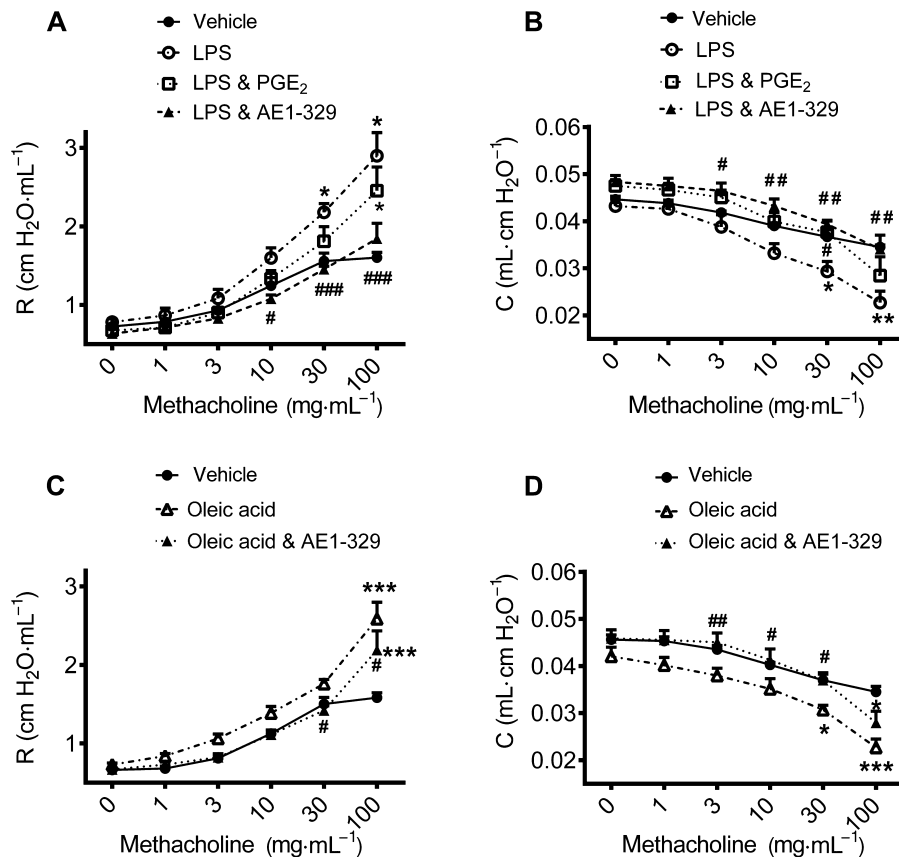


Figure 9

Lung function measured by the flexiVent system. Resistance (A,C) and compliance (B,D) induced by inhalation of increasing concentrations of methacholine was recorded 4 h after intranasal application of LPS (20 μg per mouse; A,B) and 90 min after oleic acid (0.15 $\mu\text{L}\cdot\text{g}^{-1}$ body weight; C,D) injection into the tail vein. Concomitant intranasal application of (A,B) PGE₂ (20 μg per mouse) or (A–D) the EP₄ receptor agonist ONO AE1-329 (20 μg per mouse). Data are shown as mean + SEM ($n = 6$ individual experiments per group). Data were analysed by two-way ANOVA for repeated measurements and multiple comparisons were calculated with Tukey's post test $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, compared with control; $\#P < 0.05$, $##P < 0.01$, compared with LPS or oleic acid.

et al., 2003; Buckley *et al.*, 2011). In contrast, the EP₄ receptor has been implicated to directly relax airway smooth muscle in humans, while the EP₂ receptor mediates the bronchodilator effect of PGE₂ in mice (Tilley *et al.*, 2003; Buckley *et al.*, 2011; Benyahia *et al.*, 2012; Konya *et al.*, 2013a), which was shown in our experiments using an EP₂ agonist. Therefore, it is likely that the beneficial effect of the EP₄ receptor agonist on LPS- and oleic acid-induced lung dysfunction was due to its anti-inflammatory properties, rather than direct bronchodilation.

In conclusion, the current study provides ample evidence that EP₄ receptor activation, concomitant with an inflammatory stimulus, strongly induces anti-inflammatory mechanisms, protects pulmonary microvascular endothelial barrier function and thereby ameliorates the pathology of pulmonary inflammation, such as neutrophil infiltration, plasma extravasation and deterioration of lung function in mice. Therefore, our data support a potential benefit for selective EP₄ receptor agonists in acute pulmonary inflammation.

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Author contributions

J. M., K. J., W. P., A. T. and T. B. performed and analysed the *in vitro* experiments. I. A. performed the immunohistochemistry. P. L., R. F. and I. L. performed the *in vivo* experiments and analysed the data. V. K., G. M., L. M. M., A. O., I. T. L., R. S. and A. H. provided intellectual input into study design,

data interpretation and contributed to the manuscript preparation. V. K., R. S. and A. H. wrote the manuscript.

Conflict of interest

A. H. has received consultancy fees from Bayer Pharma AG (Berlin, Germany).

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Supporting information

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Figure S1 Bronchoalveolar (BAL) fluid was collected 4 h post intranasal LPS (20 µg per mouse) or vehicle administration, and the numbers of (A) eosinophils, (B) monocytes, (C) macrophages, (D) T-lymphocytes and (E) B-lymphocytes were determined by flow cytometry. Each point shows the cell count from one animal. Data were analysed using one-way ANOVA and multiple comparisons were calculated with Tukey's post test. * $P < 0.05$, ** $P < 0.01$.

Figure S2 Blood was collected 4 h post intranasal LPS or vehicle administration, and the numbers of neutrophils were determined by flow cytometry. (A) Intranasal administration of vehicle (veh), LPS (20 µg per mouse) alone or together with PGE₂ (20 µg per mouse), and in mice pretreated the EP₄ receptor antagonist GW627368X (GW; 10 mg·kg⁻¹ s.c.; 30 min before PGE₂). (B) Intranasal application of vehicle (veh), LPS (20 µg per mouse) alone or together with the EP₄ receptor agonist ONO AE1-329 (concentrations are indicated). Each point shows the neutrophil count from one animal. Data were analysed using one-way ANOVA and multiple comparisons were calculated with Tukey's post test. ** $P < 0.01$, *** $P < 0.001$.

Figure S3 Neutrophil count in BAL fluid of mice treated intranasally with vehicle (veh), LPS (10 µg per mouse) or misoprostol (50 µg per mouse, s.c.) 2 h before and 10 h after intranasal application of LPS. Twenty-four hours after LPS application, mice were killed and neutrophils in the BAL fluid were determined by flow cytometry. Each point shows the

neutrophil count from one animal. Data were analysed using one-way ANOVA and multiple comparisons were calculated with Tukey's post test. $***P < 0.001$.

Figure S4 Neutrophil counts in BAL fluid of (A) mice treated intranasally with vehicle (veh), LPS (20 μg per mouse), PGE₂ (20 μg per mouse) concomitantly with LPS, or pretreated with the EP₂ antagonist PF-04418948 (PF; 10 $\text{mg}\cdot\text{kg}^{-1}$ s.c.). (B) Intranasal application of vehicle (veh), LPS (20 μg per mouse) and the EP₂ receptor agonist ONO AE1-259 (concentrations as indicated) concomitantly with LPS. Each point shows the neutrophil count in BAL fluid from one animal. Data were analysed using one-way ANOVA and multiple comparisons were calculated with Tukey's post test. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

Figure S5 TNF- α (A) and IL-6 (B) levels in the bronchoalveolar lavage (BAL) fluid of mice (A,B) treated intranasally with vehicle (veh), LPS (20 μg per mouse) or the EP₂ receptor agonist ONO AE1-259 (concentrations as indicated) concomi-

tantly with LPS. Each point shows the cytokine concentration ($\text{ng}\cdot\text{mL}^{-1}$) from one animal. Data were analysed using one-way ANOVA and multiple comparisons were performed with Tukey's post test. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

Figure S6 Isotype control for EP₄ staining. The endothelial layer of a lung vessel is indicated by the arrow. Scale bar = 50 μm . Micrograph is representative for five lungs.

Figure S7 Lung function was measured by the flexiVent system. Resistance (A) and compliance (B) towards inhalation of increasing concentrations of methacholine was recorded 4 h after intranasal application of vehicle, LPS (20 μg per mouse) or LPS concomitantly applied with the EP₂ receptor agonist ONO AE1-259 (30 μg per mouse). Data are shown as mean + SEM ($n = 6$ individual experiments per group). Data were analysed by two-way ANOVA for repeated measurements and multiple comparisons were calculated with Tukey's post test. $**P < 0.01$, $***P < 0.001$ as compared with vehicle; $###P < 0.01$, $####P < 0.001$ as compared with LPS.