Diplomarbeit

Effects of PGE_2 on the alveolar epithelial barrier function

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Abstract

Background: Impairment of the epithelial-endothelial barrier is a hallmark of acute lung injury (ALI) and acute respiratory distress syndrome (ARDS). The dysfunctional air-blood barrier does not only result in pulmonary infiltration of leukocytes, but also triggers edema formation in the alveoli resulting in respiratory failure. PGE_2, regardless of its known pro-inflammatory features, is considered to be protective in the lung (Vancheri et al., 2004). Our group recently reported about the barrier enhancing effect of the PGE_2 receptor EP4 on human pulmonary microvascular endothelial cells (Konya et al., 2012). Additionally, our preliminary observations reveal that PGE_2 is able to prevent the pathogenesis of LPS-induced acute lung injury in mice. Despite its importance, the impact of PGE_2 on the alveolar epithelial barrier function is not well understood.

Research Focus: My Master thesis aims to investigate the effect of PGE_2 on the alveolar epithelial barrier function and to identify the involved EP receptor subtype.

To this end, alveolar epithelial cells were isolated from male BALB/c mice as previously described (Corti et al., 1996; Marsh et al., 2009) and by additionally using negative magnetic separation. The obtained cells, being mainly alveolar type II (AT2) epithelial cells, were cultured on laminin 1 for six days resulting in transdifferentiation of AT2 cells towards alveolar type I (AT1)-like cells as described previously (Demaio et al., 2009). Changes of the epithelial barrier function were measured by using Electric Cell-substrate Impedance Sensing (ECIS). Phenotypic characterization and EP receptor expression of the isolated cells were assessed by using flow cytometry.

Results: The obtained alveolar epithelial cells represented 89% viability and, after six days of culturing on laminin 1, formed tight monolayers with the electrical resistance of typically 2000 Ω/cm^2. Phenotypic characterization of the isolated cells revealed minimal amounts of alveolar macrophages, endothelial cells and fibroblasts. Strikingly, upon exposure of AT1-like cells to PGE_2, the electrical resistance rapidly decreased. PGE_2 acted very potently on AT1-like cells, by inducing 20% barrier loss already at 10 nM concentration, while at 30 nM, PGE_2 caused irreversible barrier disruption. This function was mimicked by the EP2 agonist

butaprost and by the EP4 agonist ONO AE1-329. Interestingly, only blocking of EP2 receptor by using a selective antagonist PF-04418948 could reverse this barrier disrupting effect, while the EP4 receptor antagonist ONO AE3-208 did not have any influence. In accordance with these findings, AT1-like cells expressed EP2 and EP4 receptors as well as EP3 receptor.

Discussion: Taken together, PGE_2 induced disintegration of the alveolar epithelial cell barrier on an EP2 receptor-dependent manner. These findings suggest that the way PGE_2 controls the alveolar epithelial - capillary endothelial barrier might be very complex. On the circulation side the endothelial barrier is strengthened by PGE_2, while the air-exposed alveolar epithelial layer becomes more permeable, due to different EP receptor subset activation by PGE_2. Further investigations are required for establishing the physiological relevance of the EP2 receptor-mediated increase of alveolar permeability. These preliminary findings suggest that EP2 receptor antagonists might represent new therapeutic options for diseases with compromised air-blood barrier such as ALI and ARDS.

Zusammenfassung

Hintergrund: Permeabilitätsstörungen der aus Endothel- und Epithelzellen aufgebauten Blut-Luft-Schranke sind eines der Hauptkennzeichen des akuten Lungenversagens (ARDS=acute respiratory distress syndrome). Durch diese Schrankenstörung kommt es sowohl zu einer Einwanderung von Leukozyten in die Lungen als auch zu einem Ödem, was letztlich auch zur respiratorischen Insuffizienz führt. Während die entzündungsfördernde Wirkung von Prostaglandin E_2(PGE_2) im Allgemeinen sehr gut erforscht ist, finden sich vor allem in der Lunge protektive Effekte (Vancheri et al., 2004). Unsere Gruppe konnte in einer vor Kurzem erschienenen Arbeit zeigen, dass PGE_2 die Barrierefunktion von humanen mikrovaskulären Lungenendothelzellen EP4-Rezeptor-vermittelt stärkt. Außerdem zeigten weitere Experimente, dass PGE_2 in der Lage ist, die Pathogenese des LPS-induzierten Lungenversagens im Mausmodell zu verhindern. Trotz seiner Bedeutung ist wenig über die Wirkung von PGE_2 auf Alveolarepithelzellen bekannt.

Ziel: Meine Diplomarbeit widmet sich der Erforschung der Effekte von PGE_2 auf die epitheliale Komponente der alveolaren Barrierefunktion und der Identifizierung des zuständigen EP-Rezeptorsubtyps.

Zu diesem Zweck wurden Alveolarepithelzellen aus männlichen BALB/c-Mäusen nach schon beschriebenen Protokollen (Corti et al., 1996; Marsh et al., 2009) unter zusätzlicher Anwendung einer negativen magnetischen Selektion isoliert. Die isolierten Zellen, bei welchen es sich hauptsächlich um Alveolarepithelzellen Typ II (AT2) handelte, wurden auf Laminin 1 sechs Tage lang kultiviert, wodurch sie zu Typ-I-ähnlichen Zellen transdifferenzierten (Demaio et al., 2009). Änderungen der epithelialen Barrierefunktion wurden mittels Electric Cell-Substrate Impedance Sensing (ECIS) gemessen. Die phänotypische Charakterisierung und die Ermittlung des EP-Rezeptorexpressionsmuster wurden unter Anwendung der Durchflusszytometrie vorgenommen.

Ergebnisse: Am Ende der Isolierung waren 89% der Zellen noch intakt. Nach sechs Tagen, während derer die Zellen auf Laminin 1 kultiviert wurden, bildeten sie eine dichte Monolayer mit einem Widerstand von typischerweise etwa 2000 Ω/cm^2. Die phänotypische Charakterisierung zeigte minimale Kontamination durch Alveolarmakrophagen,

Endothelzellen und Fibroblasten. Interessanterweise zeigten die Zellen nach der Behandlung mit PGE_2 einen rapide abnehmenden Widerstand. Dieser Effekt erwies sich als sehr potent; es zeigte sich ein um 20% herabgesetzter Widerstand bei einer Konzentration von 10 nM und ein in weiterer Folge irreversibler Verlust der Barrierefunktion bei 30 nM PGE_2. Sowohl der EP2-Agonist Butaprost, als auch der EP4-Agonist ONO AE1-329 waren in der Lage, diesen Effekt ebenfalls hervorzurufen. Hier stellte sich heraus, dass nur die Rezeptorblockade mittels des EP2-Antagonisten PF-04418948 den Verlust der Barrierfunktion unterbinden konnte, während der EP4-Antagonist ONO AE3-208 keine diesbezüglichen Wirkungen hatte. Die Ergebnisse der Durchflusszytometrie zeigten, dass AT1-ähnliche Zellen sowohl EP2- als auch EP4-Rezeptoren exprimieren.

Diskussion: Zusammenfassend konnten wir zeigen, dass PGE_2 EP2-Rezeptor-vermittelt den Zusammenbruch der alveolaren epithelialen Barrierefunktion induzierte. Gemeinsam mit den schon zuvor erhobenen Daten ergibt sich ein sehr komplexes Bild der modulierenden Auswirkungen von PGE_2 auf die Blut-Luft-Schranke. Während PGE_2 den vaskulären Teil der Barriere stärkt, wird die epitheliale Schicht durchlässiger, was auf einer unterschiedlichen Aktivierung der verschieden EP-Rezeptoren basiert. Um die physiologische Relevanz dieser EP2-vermittelten Permeabilitätssteigerung zu verstehen, sind weitere Untersuchungen unumgänglich. Dennoch deuten unsere vorläufigen Ergebnisse darauf hin, dass EP2-Antagonisten eine neue therapeutische Alternative in der Behandlung von Lungenerkrankungen, die durch eine eingeschränkte Barrierefunktion gekennzeichnet sind oder mit einer solchen einhergehen, darstellen könnten. Dabei handelt es sich vor allem um ARDS, und als mildere Form, ALI (Acute Lung Injury), aber auch Asthma oder Lungenödeme gehören in diese Kategorie.

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1 Introduction

1.1 Alveolar epithelial cells

The alveolar epithelial cells line the most distal part of the airways, the alveoli. Collaborating with the endothelial cells, they form the blood-air barrier and are therefore crucial in enabling gas exchange via the alveolar capillary membrane. Furthermore they maintain the blood-air barrier by preventing airborne pathogens and particles from entering the blood stream. As the O_2 and CO_2 exchange is mediated by passive diffusion, it is also important to keep the barrier very thin, because the diffusion-capacity correlates inversely with the increasing diameter of the membrane. The alveolar capillary membrane needs to be thin, but also strong enough to maintain structural integrity, which is described as a "laminated tripartite design" (Maina et al., 2005). Consequently, alveolar epithelial cells share a single basement membrane with endothelial cells, so that diffusion can effectively take place. There are two types of alveolar epithelial cells, type I (AT1 cells) and type II pneumocytes (AT2 cells).

1.1.1 Type I alveolar epithelial cells

AT1 cells form about 95% to 99% of the cellular layer lining the alveoli, while making up no more than 10% of the total alveolar cell count. They are large in size, flat and squamous in shape in order to enable gas exchange, and are connected to each other by tight junctions. They also possess microvilli, structures rich in mitochondria showing signs of high metabolic activity. AT1 cells are closely linked to the endothelium of the pulmonary capillaries and, in contrast to other organs, these two cell types share one single layer of basal lamina (Crapo et al., 1982; Lüllmann-Rauch et al., 2008).

For a long time it was believed that AT1 cells cannot proliferate anymore, but recent studies (Dobbs et al 2009) showed their proliferative capacity *in vitro*, suggesting the same function *in vivo*. In addition, AT1 cells proliferate at very low seeding densities as compared to AT2 cells and AT1 cells are also able to form colonies. AT1 cells express octamer binding

transcription factor 4A (OCT-4A) a marker commonly associated with stem cells. AT1 cells can express markers of AT2 cells (surfactant protein C (SPC)) or Clara Cells (CC-10) when cultured under certain conditions (Gonzalez et al., 2009).

Partly, these recent advances are due to the former difficulties in isolating and culturing AT1 cells, a problem that has only been solved by use of fluorescence activated cell sorting (FACS)-based methods (Dobbs et al., 2010; Gonzalez et al., 2013). Previous models for studying the alveolar epithelium used the ability of AT2 cells to differentiate towards an AT1-like phenotype, when cultured under appropriate conditions. Those cells are similar to AT1 cells but not identical, especially considering their ion transport mechanisms (Dobbs et al., 2010).

As recently discovered, AT1 cells are important for the cleaning of fluid from the alveolus via different mechanisms, such as Na^+-channels and aquaporin (Dobbs et al., 2007; Dobbs et al., 2010).

It has been shown, that AT1 cells are also able to produce various cytokines, such as TNF-alpha and IL-6, and it has been suggested that, while AT1 cells rather promote inflammation, AT2 cells act contrarily (Wong et al., 2013).

1.1.2 Type II alveolar epithelial cells

AT2 cells are cuboidal cells mainly located at the edge of the alveoli, in a position where they do not interfere with the gas exchange. AT2 cells have lamellar bodies intracellularly, containing mainly phospholipids; and at the apical side microvilli are presented (Lüllmann-Rauch et al., 2008) .

Their major function is surfactant production what prevents the alveoli from collapsing and thereby reducing the pulmonary resistance. Defects in this function, as seen in prematurely born infants, lead to infant respiratory distress syndrome (IRDS), a very common condition in infants born before the 27^{th} week (up to 80% of those children will develop IRDS), as the AT2 cells do not start surfactant production earlier than 30 weeks of gestational age (Behrman et

al., 2007). There are various subtypes of surfactant proteins (A-D), but only surfactant protein C (SP-C) is unique to the AT2 cells and can therefore be used as a reliable marker. It is a very widespread and highly conserved protein among vertebrates and especially mammals. Surfactants (mainly form A and D) also play a role in host defense by binding to certain sugars on the surface of pathogens and thereby opsonizing them and rendering phagocytes able to clear them (Wright, 2004).

Moreover, AT2 cells prevent lung edema and maintain fluid balance by clearing excessive amounts of fluids from the alveoli, and also modulate the milieu concerning serum proteins, pH-values or ion concentration in the hypophase. This is a thin aqueous lining between the alveolar epithelial cells and the surfactant layer, and is considered as a place where modulation of surfactant proteins takes place. Moreover, it is postulated that this phase acts as “medium” for macrophages and reaction milieu for extracellular biochemical reactions (Fehrenbach, 2001).

AT2 cells are also considered as stem cells of the alveoli, as they are capable of replacing AT1 cells and also renew themselves. However, there have been certain doubts concerning their role as only progenitor cell of the alveoli, as it had recently been shown, that also AT1 cells are able to divide and proliferate, at least *in vitro* (Gonzalez et al., 2009). Nevertheless, considering the works of Uhal et al. (Uhal, 1997), it is still likely that they play the major role in repairing the epithelium after injury. There is also some evidence that bone marrow-derived cells play a role in this process (Kotton et al., 2004). As Fujino et al. could show, there is another population of stem cells present in the adult human lung, which has the capacity to form colonies *in vitro* and to transdifferentiate towards AT2 cells under appropriate conditions (Fujino et al., 2011).

They are engaged in the immunology of the alveoli; for example, it has been shown that AT2 cells synthetize lysozyme in rats, but not in humans, a protein playing a role in defense against bacteria (Fehrenbach, 2001; Singh et al., 1988). In addition, AT2 cells seem to be involved in leukocyte migration via release of arachidonic acid and interactions with endothelial cells and they are also associated with chemotaxis and stimulation of alveolar

macrophages. On the other hand, they also show anti-inflammatory properties, such as the inhibition of lymphocyte proliferation or neutrophil immigration. These functions may well be in context with their role as the “defender of the alveolus” (Fehrenbach, 2001).

1.2 Pulmonary Inflammation

Pulmonary inflammation is a major cause and underlying phenomenon of morbidity among certain populations, be it linked to asthma, COPD or to more acute conditions, such as pneumonia or acute respiratory distress syndrome (ARDS), previously also known as acute lung injury (ALI) in its less severe form. Although the relevant mechanisms are not completely the same in chronic and acute disorders, they still share the restriction of gas exchange.

With COPD being the third most likely cause of death in 2020 (Firlei et al., 2007), and ARDS showing a very high mortality rate of approximately 42-47% (Villar et al., 2011) it becomes clear, why pulmonary inflammation is still a vibrant field for research, also given the fact, that there is presently no specific treatment for ARDS.

Many cells are involved in these complex mechanisms including alveolar macrophages and alveolar epithelial cells as the first line of the innate immune response. Both cell types are rich in pattern recognizing Toll-like receptors, and are therefore crucial in the initiation of immunological pathways (Raoust et al., 2009).

1.2.1 Role of AT1 and AT2 cells in pulmonary inflammation

Several functions of alveolar epithelial cells are important in lung inflammation, mainly their role in cytokine production and the epithelial barrier function, but the fluid clearance function is also notable. Interestingly, there is evidence, that alveolar epithelium is partly spared in mild ALI and is still able to perform basic functions such as edema clearance, although permeability is increased (Wiener-Kronish et al., 1991; Zemans et al., 2004).

1.2.1.1 Barrier function

The alveolar epithelial barrier function is crucial in the prevention and clearance of lung edema and in maintaining alveolar gas exchange, thereby providing sufficient oxygen supply for the whole organism. In case of infections, the bioelectrical properties of this barrier get dysfunctional, a process mediated by TNF-alpha (Zhang et al., 1997). An impaired barrier function as induced by LPS also facilitates the migration of immune cells through the endothelial barrier, although the role of the epithelial cells remains unclear (Reutershan et al., 2006).

1.2.1.2 Chemokines and cytokines

AT2 cells have been shown to express a variety of cytokines such as TNF-alpha, IL-6, IL-1β, monocyte chemoattractant-protein (MCP)-1, growth related oncogene (GRO)-α, macrophage inflammatory protein (MIP) 1α, and granulocyte-macrophage colony stimulating factor (GM-CSF) upon injury or under pro-inflammatory conditions (Paine et al., 1993; Standiford et al., 1991; Vanderbilt et al., 2003). However, recent studies showed that the actual levels of cytokines were overestimated because of contamination with macrophages (Wong et al., 2013). AT1 cells can also produce TNF-α, IL-6 and IL-1β, however, at much higher levels than AT2 cells. Still, AT1 cells are not as thoroughly investigated, as only recent FACS-driven isolation methods were successful in isolating them in sufficient purities (Wong et al., 2013).

1.2.1.3 Fluid/Edema clearance

As edema clearance is of great importance in the outcome of ARDS, its mechanisms have been studied extensively. It was shown that the fluid which enters the lungs via the disrupted alveolar-capillary-barrier under inflammatory processes is mainly absorbed in the alveoli, but also the distal airways are partly affected (Matthay et al., 1996). AT1 and AT2 cells play a significant role under these circumstances, and although previously underestimated, AT1 cells (due to the vast surface area they cover) seem to be at least as important as AT2 cells (Dobbs et al., 2007). The fluid clearance is mediated via amiloride-sensitive epithelial Na^+-channels (ENaC) which consist out of three different subunits, being prominent in early developmental states. Amiloride-insensitive Na^+-channels are also

involved in fluid clearance, but they are more important in the adult organism. Both of these channel types are located at the apical side of the cell (Dobbs et al., 2007). For the passive uptake of sodium into the cell, Na^+/K^+-ATPase is needed to produce a sufficient concentration gradient and is therefore found on the basal side. When Na^+ is cleared from the alveolar fluid, water follows passively caused by the sodium gradient via aquaporins, and aquaporin-independent pathways, thus the edema is resolved (Zemans et al., 2004). Interestingly, in ALI, the alveolar lung fluid clearance can be reduced or increased, most likely depending upon the severity of the injury (Pittet et al., 1995; Wiener-Kronish et al., 1991).

1.2.2 Acute respiratory distress syndrome - ARDS

1.2.2.1 Definition

ARDS is a condition involving pulmonary inflammation in a diffuse alveolar damage, thus affecting not only the blood-air barrier and thereby inducing edema formation, but also, and most importantly, the gas-exchange in the lungs. As it is difficult to diagnose ARDS, there have been various algorithms for its assessment, with the most recent being the Berlin definition (2011). This consensus includes the following criteria (Fanelli et al., 2013):

- A relation to known risk factors (onset within a week of such)
- An according chest imaging (bilateral opacities, which cannot be fully explained by effusions, lobar/lung collapse, or nodules
- An investigation of the origin of the edema (hydrostatic edema, cardiac failure or fluid overload must be ruled out)
- An impairment of the oxygenation capacity, which is also used to determine the level of ARDS, from mild to severe (measured by the pO_2 in the blood/F_IO_2 at a positive end-expiratory pressure (PEEP) of 5 cmH_2O)

ARDS progresses in various phases, with different cell types primarily affected, but always impairing the “alveolar-capillary-unit” (Böcker et al., 2008):

Exsudative phase I: Mainly the endothelium is affected; an interstitial edema starts to form.

Exsudative phase II: The permeability of the epithelium increases, and epithelial cells become necrotic; alveolar edema is now present, also neutrophils are present in high numbers in the edema fluid.

Exsudative phase III: Hyaline membranes (surfactant proteins, AT2-cells, and necrotic cells) form in the alveoli.

Proliferative/regenerative phase: Endothelial cell regeneration starts, interstitial fibrosis and AT2-cell hyperplasia can be observed and edema clearance starts (although there is evidence that fibrosis is indeed an early process (Marshall et al., 1998)). This fibrosis can still be reversible under beneficial conditions (Böcker et al., 2008).

Chronic phase: This stage describes the resolution of the neutrophilic infiltration, with alveolar macrophages and mononuclear cells being present. There can be more fibrosis taking place at this time point, but in many patients, the infiltration will just be cleared (Matthay et al., 2011).

1.2.2.2 Risk factors

Risk factors are assessed based on the cause of the disease. Direct risk factors involve the

Direct risk factors	**Indirect risk factors**
Pneumonia	Non-pulmonary sepsis
Aspiration of gastric contents	Major trauma
Drowning	Severe burn
Inhalation injury	Non-cardiogenic shock
Pulmonary concussion	Pancreatitis
Pulmonary vasculitis	Drug overdose
	Multiple transfusions or transfusion associated acute lung injury (TRALI)

Table 1. Direct and indirect risk factors for the development of ARDS

lung as the organ of primary damage, while in case of indirect risk factors other underlying causes are considered (see **Fehler! Verweisquelle konnte nicht gefunden werden.** (Fanelli et al., 2013)).

1.2.2.3 Therapeutic interventions

As mentioned above and despite its seriousness, there is still no specific treatment available for ARDS. However, there are various approaches and ongoing trials discussed in a recent review (Boyle et al., 2013). A short overview will be given here, concerning the present therapy and promising investigations.

At the moment, the treatment of ARDS is mainly focused on lung-protective ventilation protocols, as non-invasive ventilation is not effective or possible in the majority of cases. Important targets here include high PEEPs (although this is discussed at the moment ((Braune et al., 2013; Fanelli et al., 2013)), low tidal volumes, low pressures and prone positioning (Beitler et al., 2014; Braune et al., 2013). Recent metaanalysis also showed beneficial effects for early neuromuscular blocking by cisatracurium, however this is part of an ongoing discussion, as the trend is rather towards early spontaneous ventilation at the moment (Braune et al., 2013; Papazian et al., 2010).

Pharmaceutical treatments are mostly restricted to the use of nitric oxids or prostaglandins as a rescue medication in hypoxemia or imminent right-sided heart failure because they act as vasodilators (Braune et al., 2013). β-agonists such as salbutamol have been investigated because of their potential to increase alveolar fluid clearance, but could not be linked to protective effects on survival or outcome and recent studies showed even increased mortality, supposedly because of their cardiac side effects (Boyle et al., 2013). Neutrophil elastase-inhibitors have also been studied, as this enzyme is known for triggering endothelial damage, but trials had to be stopped because of an increase in mortality (Boyle et al., 2013). Corticosteroids are still considered as a potential treatment as they are potent anti-inflammatory drugs, but so far, the results remain still inconsistent (Boyle et al., 2013). Statins are also currently investigated concerning their anti-inflammatory properties after a

trial showed positive effects in an LPS-induced lung injury model in healthy volunteers (Shyamsundar et al., 2009). However, further investigations are required. As fibrin is found in the alveolus during ARDS, nebulized heparin is also a potential medication and showed promising effects, triggering further investigations (Dixon et al., 2010). Platelet activation is targeted via aspirin as it also plays a role in inflammation, and a study showed preventive effects on ARDS incidence, but further confirmation studies are required (Boyle et al., 2013; Erlich et al., 2011). Other promising approaches include various growth factors, angiotensin converting enzyme blockers, vitamin D, stem cell therapy, or β-interferon (Boyle et al., 2013).

1.3 Prostaglandins

Prostaglandins (PGs) are derivatives of the arachidonic acid, produced via the cyclooxygenase-pathway, and were first isolated from seminal fluid and at that time being considered as exclusive products of the prostate gland (Goldblatt, 1935). Later on, their strong vasoactive properties were discovered, and PGs were found to play important roles in many different tissues throughout the whole body. Like the whole family of eicosanoids, they act mainly in an autocrine and paracrine fashion, meaning that they have effects on the releasing cells themselves or neighboring tissues.

The synthesis of prostaglandins starts with the release of arachidonic acid from membranes by phospholipases (mainly cytosolic PLA_2). Arachidonic acid is processed to PGG_2 first and then to PGH_2 by prostaglandin H synthase (PGHS), commonly referred to as cyclooxygenase (COX). This enzyme is an important target for a group of anti-inflammatory drugs, known as COX-inhibitors. There are two isoforms of COX; COX-1, being responsible for the basal PG secretion and COX-2, which is upregulated in case of cellular stress, such as inflammation, although there are exceptions to this general rule. Furthermore, there is also a third enzyme, COX-3, a splice variant of COX-1.

PGH_2 is then further converted into different metabolites via PG synthase enzymes, depending on the cell type and current tissue environment. In endothelial cells, mostly PGI_2 is synthetized; thromboxane is produced in platelets and macrophages, PGF synthase is found in the uterus, whereas PGD_2 is a product of mast cells and also found in the brain (Funk, 2001).

The most abundant prostaglandin remains PGE_2, which is also highly expressed in the lungs. It is synthetized from PGH_2 by either cytosolic (cPEGS) or microsomal/membrane linked PGE_2 synthase (mPEGS-1 and mPEGS-2) (Samuelsson et al., 2007). The enzyme mPEGS-1 seems to be mainly upregulated under conditions that also favor the expression of COX-2, while mPEGS-2 is constitutively expressed in many tissues. Under pathologic circumstances, such as cancer or inflammation, mPGES-2 can be also enhanced. Therefore, mPEGS-1 is a possible future target enabling more selective anti-inflammatory therapy, and also mPEGS-1 deficient mice show reduced inflammatory responses (Trebino et al., 2003).

PGE_2 is either actively transported through the membrane by the ATP-dependent multidrug resistance protein 4 (MRP-4) or diffuses passively. PGE_2 binds to specific receptors, and is rapidly metabolized by one of three cytosolic enzymes (Legler et al., 2010).

1.3.1 PGE_2 in the pulmonary inflammation

Contrary to its thoroughly investigated pro-inflammatory role in many other tissues, PGE_2 is of great importance as a limiting factor for immune response in the lung (Vancheri et al., 2004). Under physiological conditions, PGE_2 levels in the lung and especially in the epithelium of the lower respiratory tract are much higher than in plasma (Ozaki et al., 1987). It has been shown, that it plays an important role in wound healing of airway epithelial cells, especially in the early wound closure phase (spreading and migration), with the remarkable fact that COX-2, the isoform mainly associated with inflammation is also involved in this process (Savla et al., 2001). Furthermore, PGE_2 is known for its preventive effects in asthma, where it not only reduces the production of pro-inflammatory cytokines, such as histamine, leukotrienes or other prostaglandins, but it also prevents airway hyper-responsiveness by

inhibiting immune cell migration (Vancheri et al., 2004). Most likely, it also inhibits Th2 differentiation *in vivo* and COX (particularly COX-1)-deficient mice show enhanced Th2 cell count and IgE in BAL and histopathologic alterations in the lungs (Gavett et al., 1999).

Accordingly, PGE_2 is further released in the airways in response to inflammatory stimuli, such as trypsin via the protease-activated-receptor-2 and TGF-beta, secreted upon damage of epithelial cells or by immune cells (Henry, 2006; Vancheri et al., 2004).

PGE_2 is responsible for the balance of epithelial repair and inhibition of fibrosis in lung injury. While acting generally as anti-proliferative on fibroblasts, it inhibits the transition of fibroblasts to myofibroblasts, a process known to be involved in bronchial remodeling in diseases such as asthma or pulmonary fibrosis. In addition, PGE_2 has been shown to downregulate pro-inflammatory cytokines, such as IL-8, IL-12, MCP-1 and GM-CSF. GM-CSF is of great importance for the ability of fibroblasts, macrophages and alveolar epithelial cells, to produce PGE_2 and thus inhibit fibrosis and balancing wound healing (Moore et al., 2000). Expression of MHC class II and CD40 (a co-stimulatory protein) on macrophages is also downregulated by PGE_2 via a mechanism involving IL-10. This is important, because various diseases such as lung fibrosis, bronchial asthma or hypersensitivity pneumonitis are also characterized by an enhanced expression of CD40 (Vancheri et al., 2004).

It is known that PGE_2 plays an important role in microvascular pulmonary endothelial barrier function. Activation of EP4 receptor enhances the endothelial barrier function in a cAMP-independent manner, but rather via actin polymerization (Konya et al., 2012). Moreover, activation of the EP4 receptor accelerates wound closure and conserves junctional adhesions. As vascular leakage is especially pronounced in inflammation, this also promotes the importance of the immune modulatory nature of PGE_2 in the lungs. Furthermore, PGE_2 inhibits neutrophil trafficking and adhesion to endothelial cells by reduced E-selectin expression (Konya et al., 2012). The same effects have also been shown for eosinophil trafficking, being a hallmark for allergic inflammation. Additionally, PGE_2 inhibits chemotaxis in eosinophils and prevents accumulation of these cells in OVA-sensitized mice (Konya et al., 2011; Sturm et al., 2008).

1.3.2 PGE_2 and the EP1-4 receptors

PGE_2 shows a remarkable functional variety; it acts as strong vasodilator, can have proinflammatory properties, being at least partly responsive for the cardinal signs of inflammation via either vasodilation (calor, rubor) or inducing hyperalgesia (dolor)[1](Stock et al., 2001). On the other hand, it also shows protective effects, for example in the kidneys, or gastrointestinal mucosa, and accordingly, some of the most common adverse effects of COX-inhibitor therapies are gastrointestinal bleeding and alterations of renal function. The wide variety of functional responses is mediated via four different G protein-coupled EP-receptors, which are differentially distributed in tissues (Legler et al., 2010).

1.3.2.1 EP1 receptor

The EP1 receptor is known to trigger an increase in intercellular Ca^{2+} that was long thought to be caused by its coupling to G_q protein and followed by activation of phospholipase C (PLC), an enzyme, which in turn releases inositol-1,4,5-trisphosphate(IP3) and diacylglycerol (DAG). Increasing concentrations of IP3 lead to the release of Ca^{2+}-ions from the endoplasmatic reticulum. However, this hypothesis is challenged now because of the weak increase of IP3, not being notable enough to account for the high increase in intracellular [Ca^{2+}] (Alfranca et al., 2006). This increase is dependent of extracellular Ca^{2+}, and most likely mediated via an unidentified G protein, which activates calcium channels (Ichikawa et al., 2010).

In the airways, EP1 promotes airway hyper-responsiveness and bronchoconstriction via neural pathways in mice (Tilley et al., 2003), and in rats it stimulates the secretion of surfactant in AT2 cells (Morsy et al., 2001). In addition, it is involved in wound closure of airway epithelial cells (Savla et al., 2001).

[1] However, IP receptor deficient mice also show reduced inflammatory pain response, suggesting that prostacyclin is involved in pain perception too (Murata et al., 1997).

1.3.2.2 EP2 receptor

The EP2 receptor is a G_s (stimulatory) coupled receptor. Activation of EP2 receptor thus stimulates release of adenylate cyclase, thereby increasing intracellular cAMP-levels. This, in turn, triggers the activation of protein kinase A (PKA), which is now able to initiate various transcription factors, e.g. cAMP responsive element binding protein (CREB), being involved in brain metabolism and aging (Jiang et al., 2013). Another possible molecular target for cAMP is Epac, a regulator protein, being of great importance in brain functions, such as neuronal regeneration, social interactions or memory (Murray et al., 2008; Ouyang et al., 2008; Yang et al., 2012). However, Epac is present in a variety of organs, such as the kidney or the heart and even involved in insulin secretion. Furthermore EP2 is known for its ability to decrease endothelial cell permeability in a PGE_2-dependent way (Bos, 2006; Farmer et al., 2001).

On the other hand, EP2 receptor has also been shown to be linked to beta-arrestin1, generally leading to receptor desensitization, but it was recently confirmed, that the EP2 receptor thereby activates a different signaling pathway, involving Src, EGFR and finally the phosphatidyl-inositol-3-kinase, suggesting a crucial role in papilloma development (Chun et al., 2009).

The role of EP2 receptor in the lung is still not completely understood, however, it seems to be important in preventing pro-inflammatory responses such as mast cell mediator release (Kay et al., 2013). The EP2 receptor is involved in stimulation, chemotaxis and transdifferentiation of fibroblasts (Li et al., 2011), airway hyper-responsiveness and bronchoconstriction (Hartney et al., 2006). Additionally, EP2 receptor actively promotes bronchodilation (Sheller et al., 2000). Interestingly, these effects are at least partly mediated by EP4 receptor in humans and rats (Buckley et al., 2011).

1.3.2.3 EP3 receptor

The EP3 receptor is a G_i (inhibitory) G protein-coupled receptor. It decreases cAMP concentration by inhibiting adenylate cyclase activation. However, EP3 can be also coupled

to other G proteins and downstream signaling pathways, remarkably showing also a modulating effect on EP2 and EP4 receptor activation, inducing superactivation of the cAMP increase mediated by EP2 and EP4 receptors (Hatae et al., 2002; Ichikawa et al., 2010). This superactivation seems to depend on the localization of the EP3 receptor in certain membrane domains, rich in cholesterol, also known as “lipid rafts”, which effect is then mediated by coupling to G_q protein (Yamaoka et al., 2009). Moreover, there are species-specific multiple splicing variants of EP3 receptor, differing in their C-terminal sequences (Ichikawa et al., 2010).

Although the same agonist binds to these splicing variants they differ in their ability to activate G proteins, specificity of G proteins, sensitivity to agonist-caused desensitization and non-agonist-induced unspecific activation (Ichikawa et al., 1997; Negishi et al., 1993).

In the airways, the EP3 receptor induces bronchodilation, inhibits allergic inflammation (Kunikata et al., 2005), and is also responsible for PGE_2-mediated vasoconstriction in the pulmonary arteries (Norel et al., 2004). Interestingly, platelet-activating factor-induced lung edema is mediated via the EP3 receptor, and upon perfusion of isolated lungs with PGE_2, there is also significant edema formation which is prevented by EP3 antagonist treatment (Goggel et al., 2002), on the contrary to the otherwise beneficial effects of PGE_2 in the lung (Vancheri et al., 2004). In EP3-deficient mice, the mortality of Streptococcus pneumoniae-induced infection was reduced, bacterial clearance was enhanced, further illustrating the immune-modulatory properties of PGE_2 (Aronoff et al., 2009).

1.3.2.4 EP4 receptor

Just like the EP2 receptor, the EP4 receptor is coupled to a stimulatory G_s protein, which in turn increases intracellular cAMP concentration. Due to this similarity it was tempting to consider the EP4 receptor as a variation of EP2, but further studies, especially concerning the downstream signaling patterns, showed differences, and established the EP4 as an independent receptor subtype (Yokoyama et al., 2013).

The signaling pathway involving adenylyl cyclase, cAMP and thus PKA is responsible for the relaxation of vasculature mediated by nitric oxide via endothelial nitric oxide synthase, but

also activates CREB. PKA-independent activation includes Epac, and AMP-activated protein kinase AMPK. Thus a delicate orchestration of these pathways is suggested being responsible for the G_s-dependent effects of the EP4 receptor (Konya et al., 2013).

Upon phosphorylation of the G protein, β-arrestin is recruited, c-Src is activated and in turn transactivates epidermal growth factor, leading to signaling through phosphatidyl-inositol-3-kinase (PI3K) and Akt (Buchanan et al., 2006; Konya et al., 2013).

Furthermore, the EP4 receptor can also be coupled to an inhibitory G protein, inducing PI3K/ERK (extracellular-signal-regulated kinase) and protein kinase C (PKC) activation (H. Fujino et al., 2006; Luschnig-Schratl et al., 2011).

However, additional pathways have been discovered, triggering further research concerning the selective nature of the various EP4-selective agonists in terms of differing selectivity for downstream signaling compared to PGE_2, its natural agonist (Leduc et al., 2009).

The EP4 receptor mediates relaxation of trachea and bronchi in rat and human, and inhibits eosinophil effector functions (Benyahia et al., 2012). Moreover, it has been shown, to inhibit proliferation and migration of bronchial smooth muscle cells (Aso et al., 2013) and to promote apoptosis in adult lung fibroblasts (Huang et al., 2009). All of these features suggest that EP4 is a possible target for future therapy in various diseases such as asthma and chronic bronchitis (Konya et al., 2013).

Recent data from our lab also show a barrier function enhancing effect in microvascular endothelial cells and protection from LPS-induced lung injury (Konya et al., 2012). These findings are consistent with the protective effects of EP4 in LPS-induced mucus secretion from airway epithelial cells (Hattori et al., 2008).

1.4 Aim of the study

As Konya et al. could recently demonstrate, PGE_2 enhances the endothelial barrier function *in vitro,* giving a further explanation for the *in vivo* observed protective effects of PGE_2 in the lung (Konya et al., 2012). The endothelial barrier enhancing effect of PGE_2 is mediated via the EP4 receptor.

The overall aim of my Master thesis was to elucidate the role of alveolar epithelial cells in this process and to assess the effects of PGE_2 on the epithelial barrier function. Previous findings suggested a role for PGE_2 in regulating the blood-air barrier; which mechanisms might further contribute to the protective effects PGE_2. This research area is particularly interesting, since in contrast to the known roles of prostaglandins in the airway epithelium and the major bronchi, little is known about the impact of PGE_2 on alveolar epithelial cells. It is known that PGE_2 is produced by the epithelial cells, being present at high concentrations, which suggests its biological importance in the lower respiratory tract (Hageman et al., 1986; Ozaki et al., 1987).

In order to address the impact of PGE_2 on the alveolar epithelial cells, first, isolation of mouse alveolar epithelial cells had to be established and optimized. This protocol was practically non-existing in our laboratory. Thus, **my first aim** was to compare published protocols and set up the most efficient mouse alveolar epithelial cell isolation method. High viability of isolated epithelial cells was extremely desired in this study, since **my second aim** was to transdifferentiate the isolated, mainly, AT2 cells into AT1-like cells. **My third goal** was then to address the impact of PGE_2 on the AT1-like epithelial cell barrier function and to pinpoint the subtype of E-type prostanoid (EP) receptor mediating the observed effects.

2 Materials and Methods

2.1 Chemicals

All laboratory chemicals were from Sigma-Aldrich unless specified. Mouse epithelial cell culture medium (including supplementary materials) was from Cell Biologics, Inc (Chicago, IL). Dispase, rat IgG2a anti-mouse CD31-PE, isotype control (PE, AF647) were purchased from BD. The Epithelial Cell Enrichment kit, Hank's balanced salt solution modified (with 10 mM HEPES) and the EasySep magnet were all purchased from Stemcell Technologies (Vancouver, British Columbia). HEPES-buffered saline solution with phenol red, Trypsin/EDTA and trypsin neutralizing solution were from Lonza (Basel, Switzerland). DNase I from bovine pancreas was from Roche (Basel, Switzerland). Pen/Strep 100x and HEPES buffer solution 1M were from PAA. DMEM high glucose was purchased from Gibco (Carlsbad, CA). The murine IL-6 ELISA development kit was purchased from Peprotech (Rocky Hill, NJ).

The EP receptor agonists (sulprostone, butaprost, 17-phenyl trinor-PGE_2) and all rabbit anti-human polyclonal EP antibodies (EP1-4, also recognizing mouse epitopes) are from Cayman Chemicals (Ann Arbor, MI), the rabbit isotype control antibody is from Abcam (Cambridge, UK) and the secondary antibody (goat-anti rabbit AF488) is from Invitrogen (Carlsbad, CA). FIX & PERM® Cell Fixation & Cell Permeabilization Kit was purchased from Life Technologies (Carlsbad, CA), fixative solution was prepared by dissolving 1 ml CellFix in 10 ml distilled water and 30 ml FACSflow, antibody diluent was purchased from Dako (Glostrup, Denmark). Rat IgG2a anti-mouse CD326 (Ep-CAM)-APC and the anti-mouse CD11c-AF488 were purchased from eBioscience (San Diego, CA). Rat IgG2a anti-mouse ER-TR7-AF647 was from Santa Cruz (Dallas, TX). The EP4 agonist ONO-AE1 329 and the EP4 antagonist ONO-AE3 208 were kind gifts from ONO Pharmaceuticals. Ketasol (ketamine 100 mg/mL) was from aniMedica (Senden-Bösensell, Germany), and Rompun (xylazine 20 mg/mL) was from Bayer (Leverkusen, Germany).

2.2 Alveolar epithelial cell isolation

2.2.1 Obtaining single cell suspension from the mouse lung

Per isolation two BALB/c mice were euthanized with ketamine/xylazine (ketamine 10 mg/mL, xylazine 1 mg/mL in ddH2O), a cannula was inserted into the trachea and tied in place following the existing protocols with some slight modifications (Corti et al., 1996), (Marsh et al., 2009). Afterwards, the abdominal cavity was opened and the vena cava and the aorta were cut in order to let the mouse exsanguinate. After waiting for one minute, the diaphragm was cut open and the heart was exposed. The left ventricle was incised and 10 mL of HEPES buffered saline solution with phenol red were injected into the right ventricle (12 mL syringe, 25 GA needle) until the lungs were visually free of blood. The lungs were removed by cutting along the spine with the cannula still tied in place. The lungs were washed in a 50 mL falcon containing HEPES buffered saline solution. In the next step, 0.5 mL and then 1 mL of dispase was injected via the cannula into the trachea, followed by the instillation of 0.5 mL of low-melt agarose. The lungs were placed on ice for 1 minute to allow the agarose to gel, the lobes were then separated from the trachea and the heart and were then transferred to a 50 mL falcon containing 2 mL of dispase (Corti et al., 1996). After incubating the lungs for 40 minutes at room temperature, they were thoroughly minced in a 10 cm petri dish, containing 10 mL of DMEM/HEPES/DNAse (625 µL HEPES, 25 mL DMEM, 125 µL of DNAse aliquot, 250 µL of Pen/Strep, prepared in the end volume of 26 mL). After 10 min on a shaker, the cell/tissue suspension was further homogenized by pipetting up and down in a syringe (18G-needle, 20 mL) and then consecutively filtered through 100 µm and 40 µm cell strainers (VWR, Radnor, PA). The obtained single cell suspension was afterwards centrifuged (Megafuge 1.0R, Heraeus instruments) at 130 x g for 10 minutes at 8°C. After resuspending the cell pellet in cell isolation medium (10 mL HBSS/HEPES, 2% FBS, 0.1mg/mL DNAse, 100µL Pen/Strep) viable cells were counted by the trypan blue exclusion dye staining.

2.2.2 Trypan blue exclusion staining

A mixture of 4 mg trypan blue in 1 mL of PBS was used. 5 µL of cell suspension were added to 45 µL of trypan blue solution, and were afterwards counted in a Neubauer chamber. As trypan blue does not penetrate through membranes of viable cells, blue cells are considered dead, while unstained cells are counted as viable. Although it has been shown that assays using 7AAD are more reliable for determining cell viability, trypan blue was sufficient for our analysis (Xiao et al., 2003).

2.2.3 Negative magnetic selection of mouse alveolar epithelial cells

From the obtained single cell suspension the alveolar epithelial cells were isolated using the

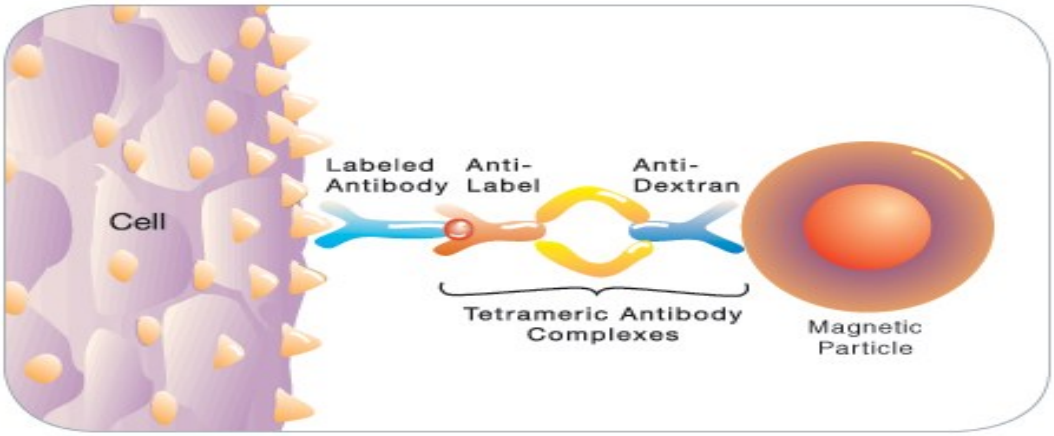

Figure 1. Principal of the negative magnetic selection. The specific antibodies are connected to the magnetic nanoparticles via the antibody complex and thereby held in the tube. (http://www.stemcell.com/~/media/Technical%20Resources/8/E/1/4/E/29165PIS_1_1_0.pdf)

Epithelial Cell Enrichment kit (see Figure 1). From this step on, cells were kept on ice.

Based on the cell count, reagents were added, and after each step, cells were incubated on ice for 15 minutes. In detail, 50 µL / 1 x 10^8 cells of the epithelial cell enrichment cocktail (containing antibodies for mouse CD45, TER119, CD31 and BP-1) was used. We used 100 µL/ 1 x 10^8 cells of the biotin selection cocktail, and 50 µL/ 1 x 10^8 cells of the magnetic nanoparticles.

The cells were then put aside in the magnet for 5 minutes followed by pouring the magnetically not separated cells off in a new tube, thereby leaving the previously marked cells in the old tube. The old tube was washed with cell isolation medium, was again put aside in the magnet for 5 minutes and poured off in the new tube. The obtained cell suspension was then centrifuged (130 x g 10 minutes, 4°C), washed and placed into the magnet, where it was again incubated for 5 minutes and finally centrifuged. The purified cells were then counted in trypan blue and seeded in the required cell density of approximately 1 x 10^6/cm² (Wise, 2002) for cell culturing and further functional experiments. Cell culture surfaces were pre-coated with laminin 1 isolated from Engelbreth-Holm-Swarm murine sarcoma basement membrane.

2.3 Cell culture

Isolated mouse alveolar epithelial cells were kept in culture for 6 to 7 days in mouse epithelial cell medium (supplemented with 0.1% EGF, 0.1% ITS, 0.1% hydrocortisone, 5% FBS, 1% L-glutamine and 1% antibiotics-antimycotics) until a tight monolayer of transdifferentiated AT1-like cells was formed. We used published protocols with slight modifications (Demaio et al., 2009). Media was not changed until the third day after seeding, but from then on every day. Afterwards, the cells were used for receptor staining, electrical resistance measuring and cytokine secretion experiments.

2.4 Epithelial electrical resistance measurements

Electric Cell-substrate Impedance Sensing (ECIS) is a highly reproducible method for determining the electrical resistance of a cellular monolayer under certain conditions. In the bottom of the wells, there are gold electrodes, which enable resistance, impedance and capacitance measurements (see Figure 2). When cells spread and thereby cover the electrodes, the resistance increases and will be measured online. If the cellular layer gets compromised (for instance after bacterial LPS treatment), the resistance decreases. For our

experiments, the ECIS Z theta instrument was used with the 96-well array station and compatible 96W20idf chips. All ECIS data shown were obtained at the frequency of 4 kHz.

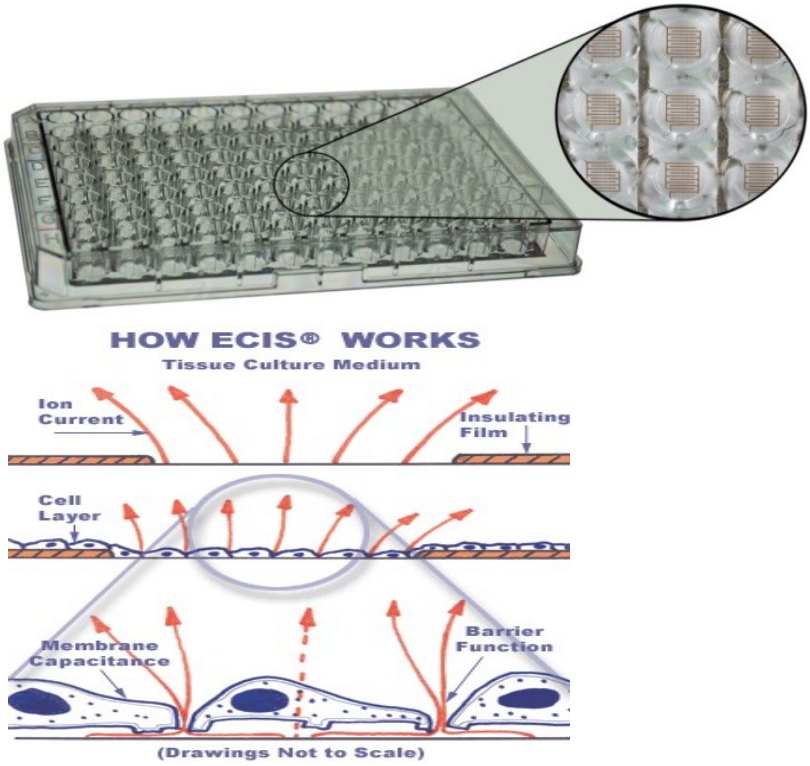

Figure 2. Measuring epithelial electrical resistance. Cells cover the electrodes and thereby increase the resistance. Thus, epithelial cell barrier function can be measured. (http://www.biophysics.com/cultureware.php)

Prior to cell seeding, chips were activated by coating with 10 mM L-cystein in destilled water, followed by coating with murine laminin in order to promote the formation of a tight monolayer (Demaio et al., 2009). Freshly isolated alveolar epithelial cells, mainly containing AT2 cells, were seeded at a density of approximately 350,000-400,000 cells per well and grown to confluence within 6-7 days. By this time, AT2 cells transdifferentiated into AT1-like epithelial monolayer forming cells. The alveolar epithelial monolayers were evaluated by phase contrast microscopy and by shortly measuring the resistance. If the cells appeared confluent and the resistance values were over 1500 ohm/cm², the experiments were performed, which was typically the case on day 6 or 7. Epithelial cells were serum-starved by using mouse epithelial cell medium with only 2% of FBS and no other supplements prior to experiment and 4 hours baseline was recorded. All substances used for resistance

measurements were diluted in the serum-starvation medium and added in a volume of 10 µL per well in the accordingly concentrated form. A pre-treatment of 20 minutes was performed for antagonists and for the cyclooxygenase-inhibitor diclofenac. Electrical resistance was recorded for 24 hours. Cellular supernatants were collected and frozen for further detection of cytokines and prostaglandins.

2.5 EP receptor staining by flow cytometry

In order to examine the expression of EP receptor subtypes, freshly isolated cells were seeded in 48-well plates, coated with laminin and grown to confluence for 6 to 7 days. The cells were harvested by first washing with HBSS and then incubating them with trypsin/EDTA at room temperature until detachment was visible. In order to neutralize trypsin, HBSS with 5% FCS was added. The cells were pooled in a falcon, the wells were thoroughly washed again and the resulting suspension was centrifuged (5 min at 220 x g, RT); all following washing steps were performed using PBS without Ca^{2+} and Mg^{2+}. The pellet was resuspended and afterwards the cell suspension was distributed into small FACS tubes. After centrifuging again (7 min at 400 x g, RT), supernatants were discarded, Fix & Perm A solution(the fixation component) was added and cells were incubated for 15 min. The same was done with Fix & Perm B solution (responsible for permeabilization), cell pellets were washed again and the desired primary antibodies solved in antibody diluent were added and incubated at 4°C for 1 hour. Fix & Perm is a ready-to-use kit, in order to permeabilize cells to gain access to intracellular structures, as for us, to the intercellular binding sites for the EP-receptor-antibodies. The used antibodies are listed in List 1.

Antibody	**Concentration**
EP1 rabbit polyclonal Ab (Stock 0.5 mg/mL)	4 µg/mL
EP2 rabbit polyclonal Ab (Stock 0.5 mg/mL)	4 µg/mL

EP3 rabbit polyclonal Ab (Stock 0.5 mg/mL)	4 µg/mL
EP4 rabbit polyclonal (Abcam) (Stock 0.3 mg/mL)	4 µg/mL
Rabbit isotype control Ab (Stock 0.2 mg/mL)	4 µg/mL
2nd goat anti-rabbit AF488 (Stock 2 mg/mL)	4 µg/mL

List 1 : Antibodies used for the EP receptor staining

The cells were then washed, centrifuged (7 min at 400 x g, RT), the secondary antibody (goat anti-rabbit AF488) was added and cells were incubated at 4 °C for 30 minutes.

Cells were washed and centrifuged again (7 min at 400 x g, RT) and fixative solution was added. Samples were measured by flow cytometry.

2.6 Profiling of isolated cells by flow cytometry

Cells from single cell suspension and the magnetically isolated alveolar epithelial cells were used for the flow cytometric profiling. Cells were transferred to 3 mL PBS-, centrifuged (400 x g, 7 minutes at room temperature), resuspended in PBS- and distributed to small FACS tubes. The cells were centrifuged again (400 x g, 7 minutes at room temperature), and meanwhile the antibodies were diluted in antibody diluents (see List 2).

The supernatants were discarded, and 100 µL of staining solution were added to the tubes. After 30 minutes incubation at 4°C, 250 µL of PBS- were added and the cells were centrifuged again (400 x g, 7 minutes at room temperature). In the next step, the pellets were resuspended in 100 µL of fixative solution and the samples were measured by flow cytometry.

Epithelial cell	
rat IgG2a anti-mouse CD326 (EpCAM)-APC	0.2 mg/mL
Macrophage	
Armenian hamster IgG Anti-mouse CD11c Alexa fluor 488	0.5 mg/mL

Fibroblast	
Rat IgG2a anti-mouse ER-TR7 AF647	0.05 mg/mL
Endothelial cells	
Rat anti-mouse CD31-PE	0.2 mg/mL
Isotype control	
Rat IgG2a anti mouse AF647	0.2 mg/mL
Isotype control	
Rat IgG2a k PE	0.2mg/mL

List 2 Antibodies used for the identification of isolated cells

2.7 Statistical analyses

Electrical resistance data were analyzed for significance by two-way ANOVA, while flow cytometric data were analyzed by t-test using GraphPad Prism 4.

3 Results

3.1 Isolation and culture of murine alveolar epithelial cells

Dispase digestion of the mouse lungs resulted in a single cell suspension containing approximately 70 million cells per mouse with around 89% viability as determined via trypan blue exclusion staining.

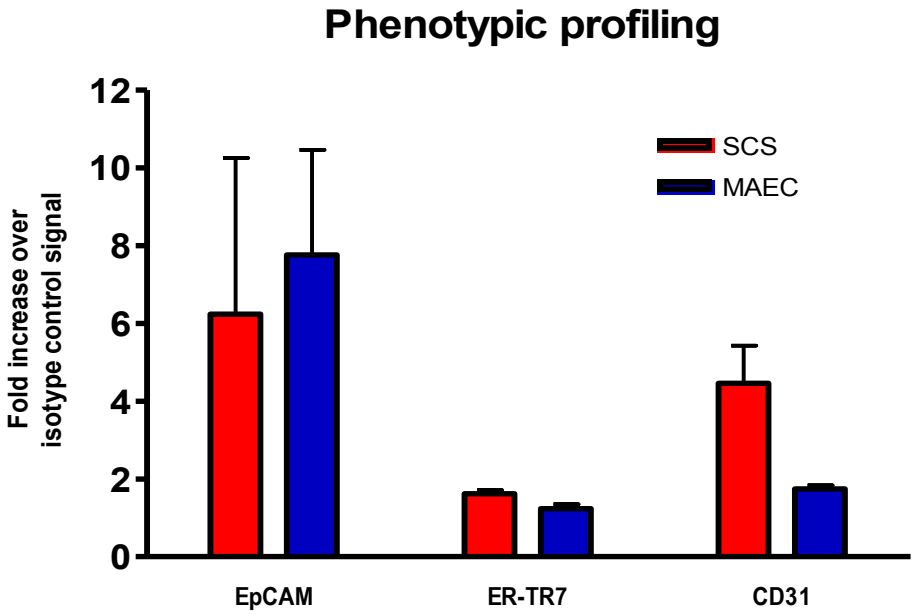

Figure 3 Phenotypic profiling of isolated cells. SCS stands for single cell suspension (gained after digestion, shown in red) and MAEC for mouse alveolar epithelial cells (obtained after magnetic separation of SCS, shown in blue). EpCAM is an epithelial cell marker, ER-TR7 identifies fibroblasts, whereas CD31 is a marker of endothelial cells. Data shown as mean + SEM, n=3.

For enrichment of mouse alveolar epithelial cells (MAEC) out of the pulmonary single cell suspension (SCS) we used a negative magnetic separation technique, which provided approximately 14 million cells with unaffected viability of 89%. In Figure 3, the results from three independent isolations are shown in terms of contaminating cells. Analysis shows a decreased presence of endothelial cells and a slightly increased signal for epithelial cells in purified MAEC cells after magnetic selection of SCS. Fibroblasts were almost not present in

either SCS or MAEC fractions. These results show that our established alveolar epithelial cell purification method is very sufficient in isolating epithelial cells out of the mouse lung.

If these purified cells were seeded on laminin 1, they formed a confluent monolayer by day 6, as demonstrated by previous studies (Demaio et al., 2009). This suggests that transition of the isolated AT2 cells towards an AT1-like phenotype was successful, and promoted by the substrate and cell culture medium used. The electrical resistance assessed by Electric Cell-substrate Impedance Sensing (ECIS) reached typical values of 1500-2300 Ω/cm² on day 6, with no further increase if cultured for longer periods of time, being comparable to the results of Demaio et al.

3.2 PGE_2 disrupts epithelial barrier function

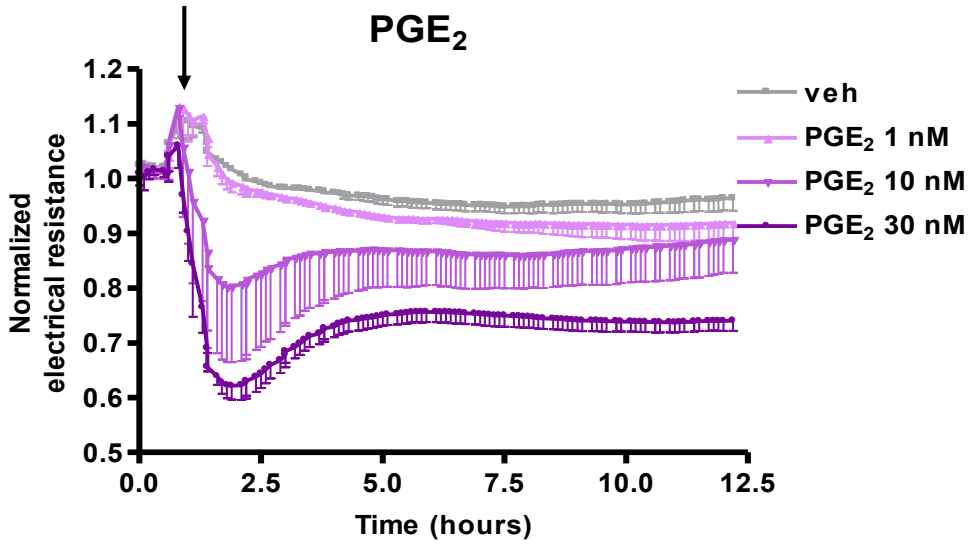

Figure 4. PGE_2 disrupts the epithelial barrier function in a concentration-dependent manner. The arrow indicates the start of the treatment. Normalized electrical resistance is shown as mean + SEM, n=3.

It was recently shown that PGE_2 enhances the lung microvascular endothelial barrier function and protects against neutrophil trafficking (Konya et al., 2012). Recent observations from our lab also showed protective effects of PGE_2 in an LPS-induced acute lung injury (ALI) mouse model. Hence, we hypothesized that PGE_2 might also affect the barrier function of alveolar epithelial cells. To this end, freshly isolated cells were grown to confluence on

laminin-coated ECIS chips. On day 6 prior to treatment with different concentrations of PGE_2, cells were serum-starved for 4 hours and a baseline resistance was recorded for 1 hour. Electrical resistance was determined using ECIS at multiple frequencies, data are shown in Figure 4. Surprisingly, PGE_2 decreased the epithelial resistance in a concentration-dependent manner with the highest effect seen at a concentration of 30 nM. We observed approximately 40% reduction of the baseline resistance, which turned out to cause an irreversible damage in the epithelial barrier function. Higher concentrations of PGE_2 did not induce further disruption of the epithelial barrier function (data not shown).

3.3 The epithelial barrier disrupting effect is modulated by the EP2 receptor

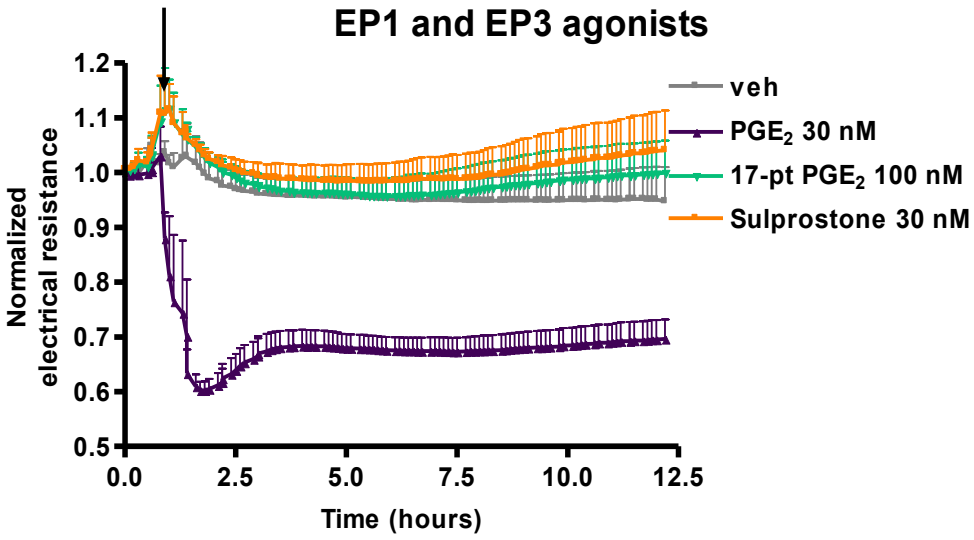

Figure 5. The EP1 agonist 17-pt PGE_2 and the EP3 agonist sulprostone did not influence the alveolar epithelial barrier function. PGE_2 treatment is used as control. The arrow indicates the start of the treatment. Normalized electrical resistance is shown as mean + SEM, n=3.

After this remarkable observation, we set out to determine the responsible receptor for this effect. As EP4 and EP2 receptors have (at least in endothelial cells) rather been shown to increase barrier properties (Farmer et al., 2001; Konya et al., 2012), we first investigated the effects of EP1 and EP3 receptor agonists (17-phenyl trinor PGE_2 and sulprostone respectively, see Figure 5). However, these selective agonists showed no effects on alveolar

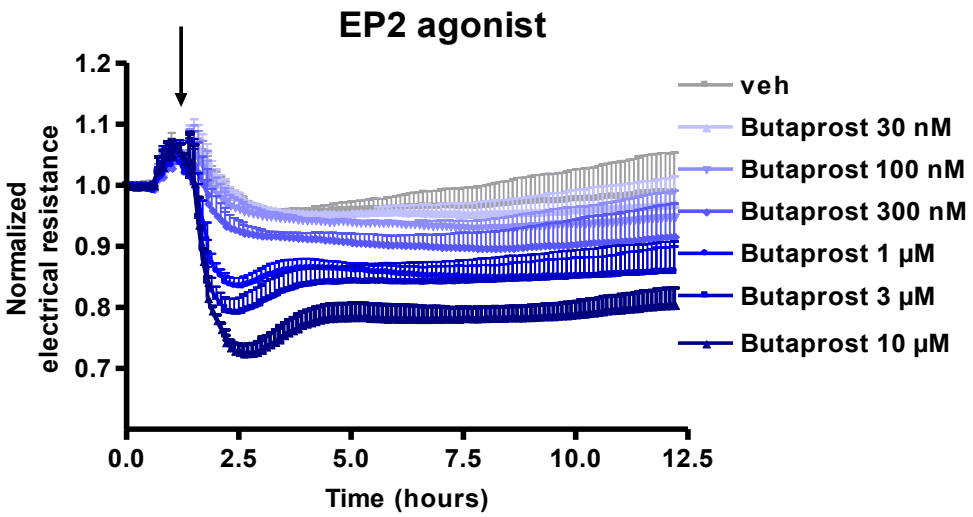

Figure 6. The EP2 agonist butaprost disrupts the alveolar epithelial barrier function. The arrow indicates the start of the treatment. Normalized electrical resistance is shown as mean + SEM, n=3.

epithelial cells except of a slight tendency to increase the resistance. Also higher concentrations showed no additional effects (data not shown). We therefore investigated the effects of a selective EP2 agonist (butaprost). Butaprost seemed to mimic the barrier disrupting effect of PGE_2, however, only at high concentrations such as 3 and 10 µM (see Figure 6). In addition we also investigated the effects of a non-selective EP1, EP2 and EP3-

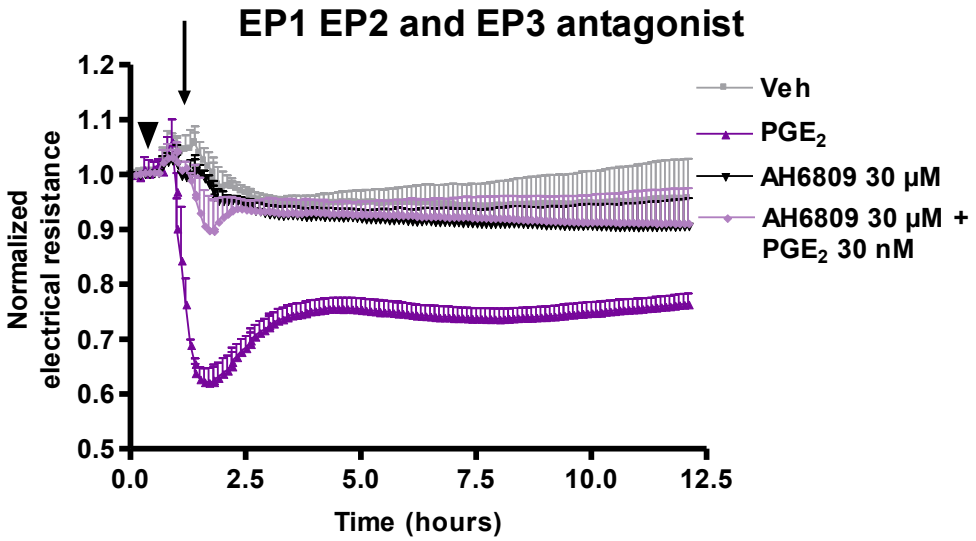

Figure 7. The non-selective EP1, EP2 and EP3 antagonist AH6809 reversed the barrier disrupting effect of PGE_2. Cells were pretreated with the antagonist for 30 minutes prior to the PGE_2 treatment. The arrowhead indicates the starting of the antagonist pretreatment, while the arrow indicates the start of PGE_2 treatment. Normalized electrical resistance is shown as mean + SEM, n=3.

antagonist (AH6809) in order to fortify our hypothesis (Figure 7), which proved effective in terms of inhibiting the effect of PGE_2. Moreover, we also used the relatively new and highly selective EP2 antagonist PF-04418948 (af Forselles et al., 2011) in order to prove specific involvement of EP2 receptor. The EP2 antagonist, PF-04418948 completely abolished the PGE_2-induced barrier disruption without having additional effects on its own (Figure 8). We can therefore conclude that the epithelial barrier disrupting effect of PGE_2 is indeed mediated via the EP2 receptor. Furthermore, PF-04418948 also abolished the effect of butaprost on the alveolar epithelial cells, as shown in Figure 9.

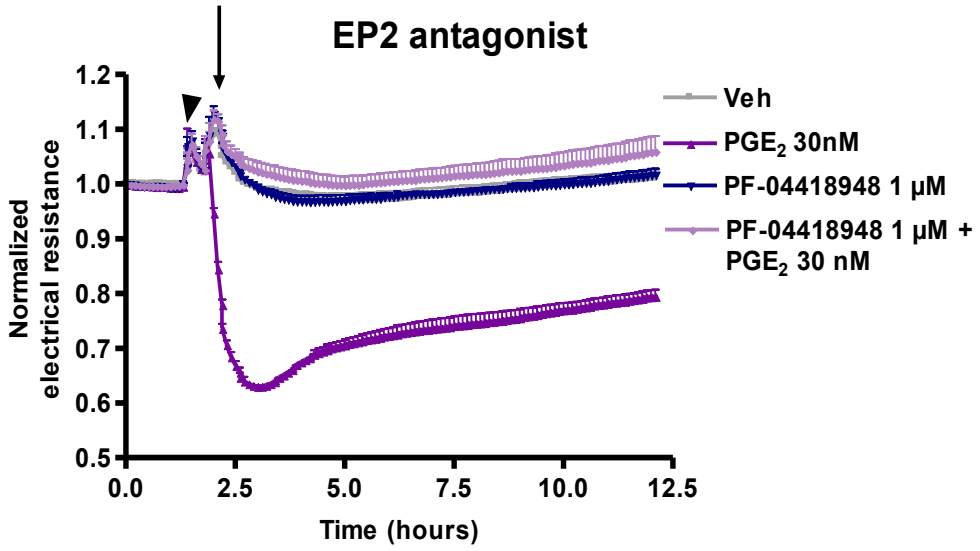

Figure 8. Effects of the EP2-antagonist PF-04418948 on the PGE_2 mediated barrier disruption. Cells were treated with the antagonist 30 min prior to the PGE_2 treatment. The arrowhead indicates the starting of the antagonist pretreatment. The arrow indicates the start of the treatment. Normalized epithelial resistance is shown as mean + SEM, n=4.

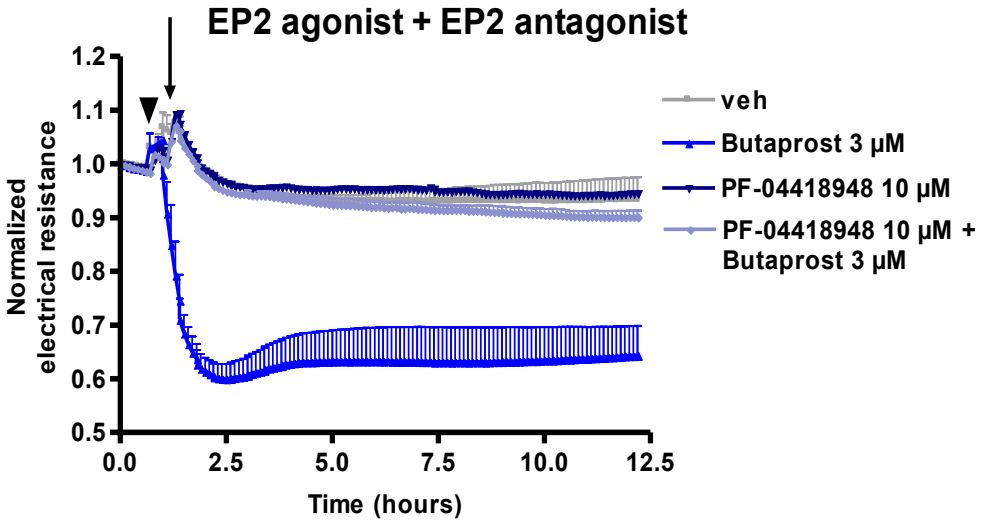

Figure 9. The selective EP2 antagonist PF-04418948 reversed the butaprost induced loss of barrier function. Cells were treated with the antagonist 30 min prior to the PGE_2 treatment. The arrowhead indicates the starting of the antagonist pretreatment. The arrow indicates the start of the treatment. Normalized epithelial resistance is shown as mean + SEM, n=3.

3.4 The EP4 agonist ONO AE1-329-induced epithelial barrier disruption is mediated via the EP2 receptor

In addition to EP1, EP2 and EP3 receptors, we also wanted to assess the contribution of EP4

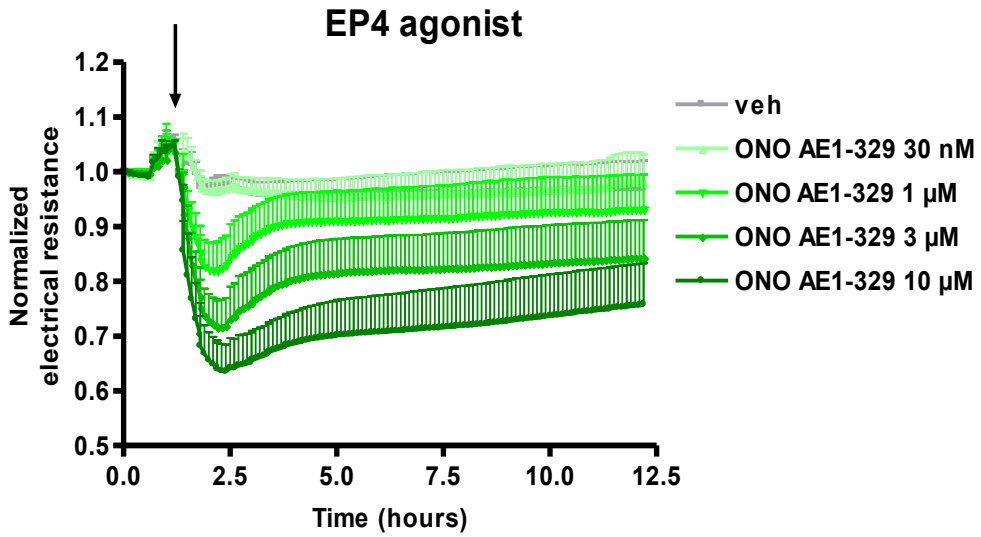

Figure 10. The EP4 agonist ONO AE1-329 decreases the epithelial barrier function. The arrow indicates the start of the treatment. Normalized electrical resistance is shown as mean + SEM, n=4.

receptor to the regulation of alveolar epithelial barrier function by PGE_2. The EP4 agonist ONO AE1-329 could induce similar epithelial barrier disruption as seen with EP2 agonist and PGE_2 itself. However, the EP4 agonist-caused decrease in the epithelial electrical resistance was only distinct at high concentrations (3 and 10 µM) (Figure 10). We observed similar phenomenon with the EP2-selective agonist butaprost, while only PGE_2 treatment induced barrier disruption very potently. In order to further test the specificity of EP4 receptor involvement, we applied a selective EP4 antagonist ONO AE3-208. Surprisingly, pretreatment of AT1-like cells with the EP4 antagonist did not prevent the epithelial barrier disruption triggered by PGE_2 treatment (Figure 11).

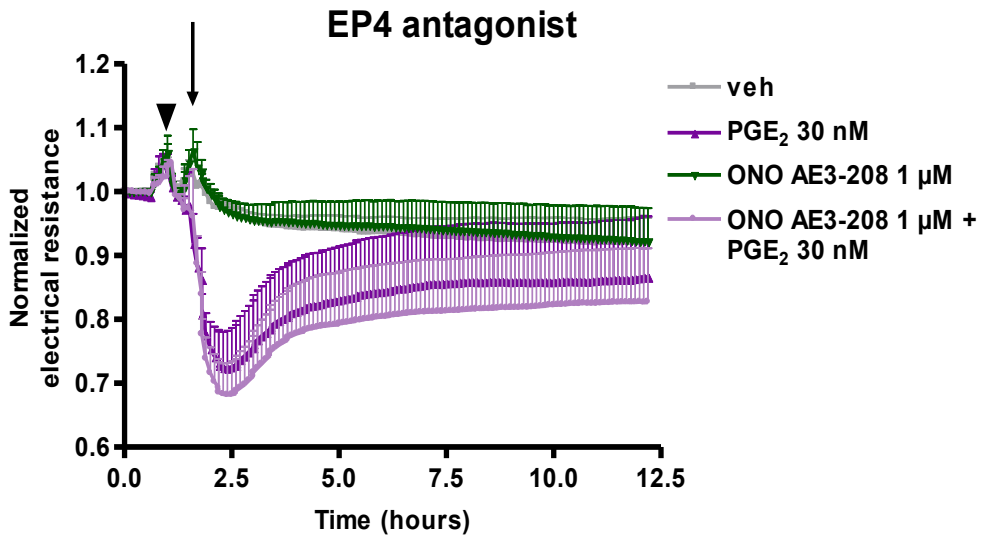

Figure 11. Blocking of EP4 receptor does not prevent the PGE_2-induced disruption of alveolar epithelial barrier. AT1-like cells were pretreated with ONO AE3-208 EP4 antagonist for 30 minutes prior to treatment with PGE_2. The arrowhead indicates the pretreatment, while the arrow indicates the start of the PGE_2 treatment. Normalized electrical resistance is shown as mean + SEM, n=4.

However, using the selective EP2 antagonist PF-04418948 prior to EP4 agonist treatment clearly prevented the EP4 agonist-induced decrease in the epithelial barrier function. These results suggest that the EP4 agonist ONO AE1-329 might have some non-specific binding affinity to EP2 receptor, since the EP2 receptor antagonist completely reversed the ONO AE1-329-triggered reduction of epithelial barrier function (Figure 12).

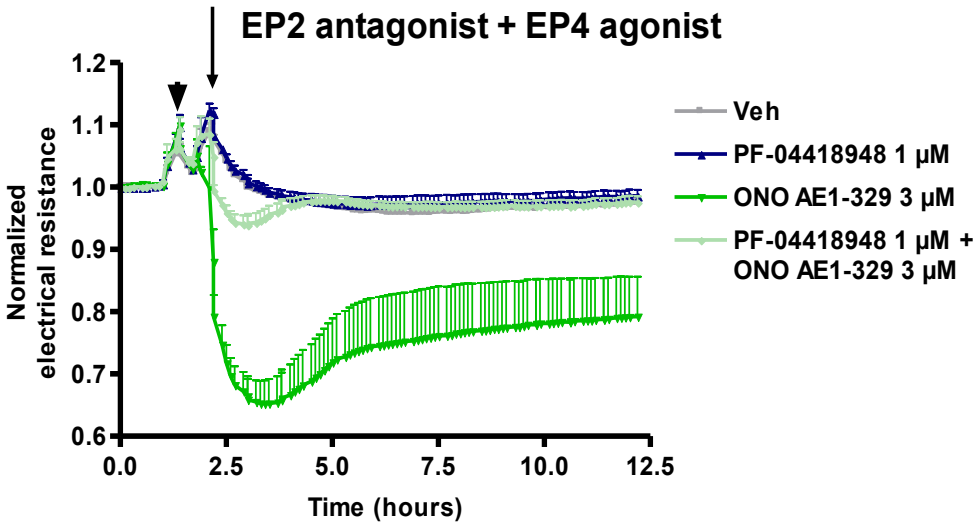

Figure 12. Selective blocking of EP2 receptor inhibits the barrier disruption caused by the EP4 agonist. AT1-like cells were pretreated with the EP2 antagonist PF-04418948 for 30 minutes prior to EP4 agonist ONO AE1-329 treatment. The arrowhead indicates the pretreatment, while the arrow indicates the start of the EP4 agonist treatment. Normalized electrical resistance is shown as mean + SEM, n=4.

3.5 LPS disrupts the alveolar epithelial barrier

As it has been described that LPS disrupts monolayers of microvascular pulmonary

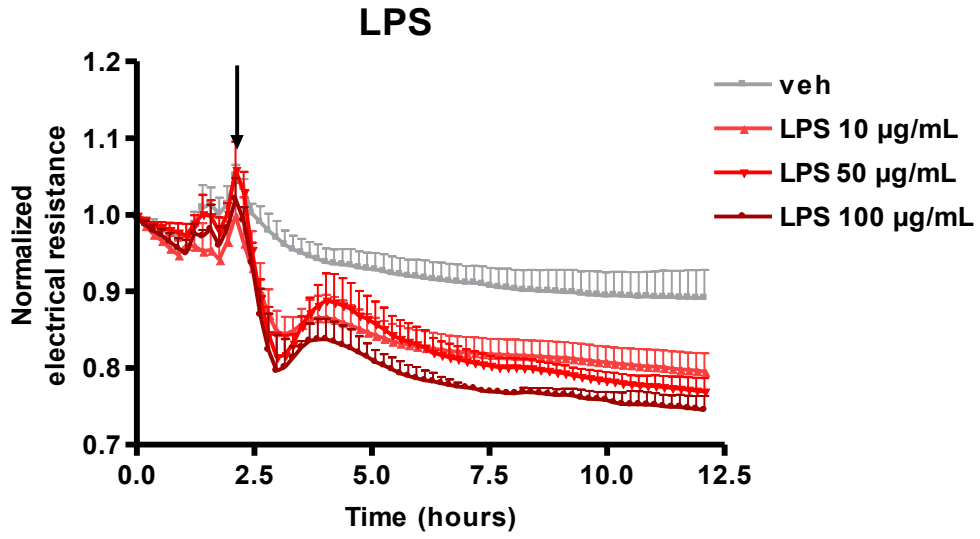

Figure 13. LPS treatment leads to decreased epithelial barrier function. The arrow indicates the start of the LPS treatment. Normalized electrical resistance is shown as mean + SEM, n=3.

endothelial cells, we hypothesized that LPS would also induce similar changes on alveolar epithelial cells (Bannerman et al., 1999), although other papers stated that it is not primarily used for modelling alveolar epithelial damage (Matute-Bello et al., 2008). We found a concentration-independent decrease in the epithelial electrical resistance. Furthermore, very high concentrations were needed to induce this effect (Figure 13).

3.6 Alveolar epithelial cells express EP receptors

We determined EP receptor expression on the purified alveolar epithelial cells by using indirect flow cytometry. Figure 14 shows that these cells on day 6 express all four EP receptors to some extent. We found that EP3 is most dominantly present on the alveolar epithelial cells but also EP2 and EP4 receptors are expressed. Very low expression level was observed for EP1 receptor. These results reveal that EP2 and EP4 receptors but also EP3 receptor are present on the AT1-like cells.

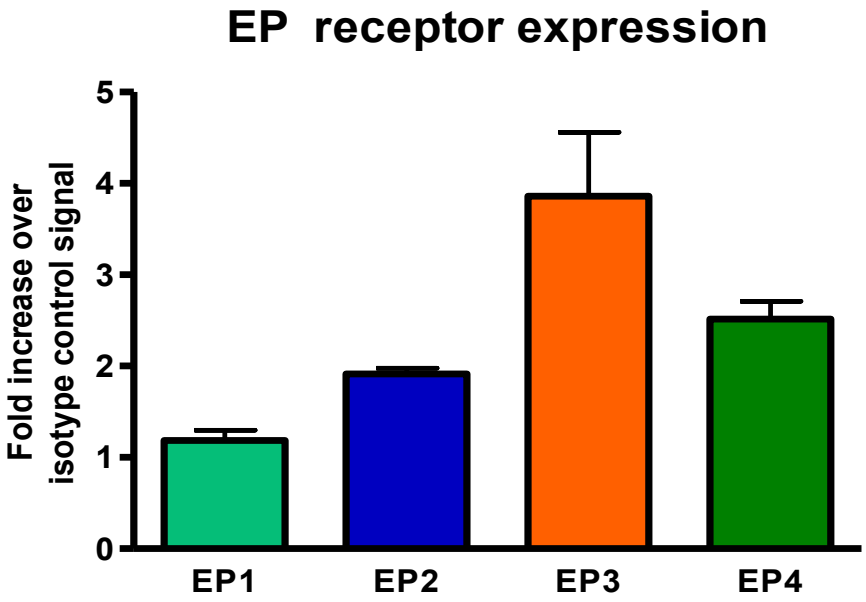

Figure 14. EP receptor expression of MAEC transdifferentiated into AT1-like cells. Specific expression of the EP receptors was assessed by using indirect flow cytometric analysis. Data are shown as mean + SEM, n=4.

4 Discussion

As the epithelium lining the alveoli is one of the most affected areas in ARDS with substantial necrosis of alveolar epithelial cells and edema formation, it is of great importance to have a reliable model for in vitro experiments (Böcker et al., 2008). Common approaches include application of immortalized cell lines, such as the AT2-like cell line A549, a human cancer cell line, or immortalized mouse cells, but do not have the benefits of primary cells. Most studies used larger animals such as rats (Dobbs, 1990), in view of the higher number of cells obtained after the isolation. However, not every laboratory has a rat-adapted facility, and mice are a more common object of studies in many fields. So, especially considering cross-talk to murine endothelial cells, we established, according to the methods of previous works (Corti et al., 1996; Demaio et al., 2009; Marsh et al., 2009) a protocol for the isolation of high numbers of viable alveolar epithelial cells from Balb/c-mice, using a method of magnetic separation, which made it possible for us to further study the effects of various substances on a monolayer of type-1-like cells. Although other authors suggest that the application of laminin 5 would be more efficient in regards of higher transepithelial resistance (Demaio et al., 2009), laminin 1 (which is more accessible and inexpensive) has proved to be sufficient in terms of maintaining a stable monolayer with reasonably high resistance.

Future applications of the isolated cells also include co-cultures with endothelial cells, for example on transwell filters, which would provide us with an even more relevant model to investigate immune cell migration. As the majority of current experiments covers only one specific cell type (e.g. endothelial cells), this would be an important progress in better understanding the *in-vivo* processes in the alveoli under inflammatory conditions.

As PGE_2 is the most abundantly expressed prostaglandin in humans, and the more so in the lungs (Ozaki et al., 1987; Samuelsson et al., 2007), PGE_2 has been a promising target for research on lung-resident cells. We hoped to elucidate the role of PGE_2 in the distal airways, namely in the alveoli. As we could show in our study, in great contrast to the effects on the endothelium (Konya et al., 2012), PGE_2 disrupts the epithelial barrier function in murine type-1-like cells. Similar effects have been observed by other groups recently (Su et al., 2013), although so far only in human lung epithelial cells. In other studies, PGE_2 also showed

resembling epithelial barrier disrupting effects in Caco2-cells, a human colorectal adenocarcinoma cell line, where this was mediated by EP1 and EP4 receptors, but not by EP2 receptor (Rodriguez-Lagunas et al., 2010). A barrier-modulating effect of PGE_2 could be shown in Madin-Darby canine kidney cells (Flores-Benitez et al., 2009). This barrier disrupting effect in the lung was especially surprising, as PGE_2 has been shown to be a protective agent *in vivo* in LPS-induced acute lung injury and did not only decrease edema fluid, but also inhibited neutrophil trafficking and thus targeted hallmarks of pulmonary inflammation. An explanation could include the fact that LPS does not as much promote epithelial damage but rather affects endothelial cells (Matute-Bello et al., 2008), so it could be possible that the protective effects in endothelial cells exceed its barrier disrupting effect on the epithelium. Further studies are required in order to quantify the effects of PGE_2 in experimental settings with more pronounced epithelial damage. Another explanation could also refer to the classical model of disease progression in ARDS, where early stages include the endothelium, while the epithelium is affected only later (Böcker et al., 2008), meaning that the barrier disrupting effects could only be relevant at a later time point, not measured in the experiments in our lab so far (mice were sacrificed in the *in vivo* experiments after 4 hours). However, this seems unlikely, as the classical model with exactly distinguishable stages remains all but undisputed (Marshall et al., 1998), and as epithelial damage can be clearly observed in other ALI-models (such as acid aspiration) after 2 hours (Matute-Bello et al., 2008). It would be important to further investigate this mechanism by staining the lungs of PGE_2-, LPS- or PGE_2 + LPS-treated mice in order to investigate the morphologic changes is various cell types according to the treatments.

We could also show that the obtained cells do in fact express EP receptors, therefore confirming the data from our subsequent experiments regarding the effects of prostaglandin E_2 and its agonists on these cells. It would be of further interest, as it has been previously shown that especially in the cells of the airways, human and murine EP-receptors differ concerning their relevance for certain effects of PGE_2 (Konya et al., 2013), to investigate the type of EP receptor responsible for the observations made by Su et al.(Su et al., 2013). In this paper, human alveolar epithelial cells were cultured on transwell filters and treated with bile

acids, as a model for ALI. They could show that the barrier disrupting effect of bile acids was mediated via PGE_2, cPLA, COX2, C-jun N-terminal kinase (JNK), and p38. However, no EP receptor subtype had been identified to our knowledge.

As further explained above, epithelial barrier disrupting effects of PGE_2 have so far been associated with EP1 and EP4 receptors, which were not responsible for the observed alterations in our experiments (Rodriguez-Lagunas et al., 2010). As we could show, both a non-selective and a selective EP2 antagonist were able to prevent PGE_2-induced epithelial barrier disruption while butaprost mimicked the effects of PGE_2 (an effect also prevented upon treatment with the selective EP2 antagonist). Although it seems reasonable to conclude that the EP2 receptor is responsible for the effects of PGE_2, there are still some uncertain components in our study. First of all, the high concentrations of butaprost needed for inducing PGE_2-like effects are still not completely understood, as it is rather used in lower concentrations than PGE_2 to provoke the same actions. It would therefore be important, to repeat the experiments with possibly more potent EP2 agonists. Furthermore, we do not know up to now, which changes on the cellular level are responsible for the increase in permeability, as the ECIS device does not distinguish whether cells perish, contract or proliferate, as long as the electrical resistance changes. It would be helpful to visualize the cells after the treatment to better understand these changes, although it is likely that the loss of tight junctions, as described by Su et al. plays a major role in this process.

Another interesting observation in our work was the non-selective activation of the EP2 receptor via the EP4 agonist ONO AE1-329, which had not been shown in the literature so far. Because its actions were blocked by using of the selective EP2 antagonist, we concluded that the high concentrations used, seemed to allow unspecific binding, rather than suggesting a role for the EP4 receptor. However, it would be of great interest to examine the effects of the EP4 agonist on human cells.

As PGE_2 is known to be expressed in the lungs, we also measured its levels by means of ELISA in the supernatants of alveolar epithelial cells and could thereby show that it is in fact present at high concentrations (up to 4000 pg/mL at steady state and up to 6000 pg/mL

upon stimulation with LPS 10 µg/mL, data not shown) and that its secretion is inhibited by diclofenac. As we hypothesized that the barrier disrupting effect of LPS could, similarly to the findings of Su et al. (Su et al., 2013), depend on PGE_2, we inhibited COX with diclofenac (data not shown). However, this did not alter the decrease in resistance upon LPS treatment, suggesting a different pathway for the actions of LPS in contrast to those of bile acids, which were very effectively abolished by diclofenac. It is interesting that endogenous PGE_2 does not seem to alter the alveolar epithelial barrier function, but shows otherwise remarkable effects. It seems likely that endogenous PGE_2 is involved in the cross-talk between epithelium and endothelium in the lung and that the raise of PGE_2 levels following LPS stimulation could be associated with the protective effects of PGE_2 on microvascular endothelial cells. This assumption is supported by recent findings from our lab, showing no PGE_2 in supernatants from endothelial cells and only low levels in alveolar macrophages. Although this deduction seems reasonable enough, the final evidence that alveolar epithelial cell-derived PGE_2 influences endothelial cells *in-vivo* is still lacking. However, also the recent observations of Wang et al. (Wang et al., 2013) provide strong evidence for this theory, as in co-cultures of alveolar epithelial and pulmonary microvascular cells, epithelial cells had strengthening effects on the endothelial barrier function upon LPS-stimulation. This effect was also mimicked by only using epithelial cell culture supernatants. Taken together, this could be a possible explanation for the significance of the PGE_2, although experiments are still in progress in our lab.

Alveolar epithelial cells also produced interleukin-6 upon stimulation with LPS 10 µg/mL (typically at a level of 1000 pg/mL). This effect was partly inhibited by pre-incubation with diclofenac, which suggests that the release of IL-6 is prostaglandin-dependent (data not shown). This is of special interest, because it is known that IL-6 plays a crucial role in alveolar barrier dysfunction and permeability increase in ventilator associated lung injury (Gurkan et al., 2011). So far, the responsible receptor subtype has not been identified, but as mentioned below, selective blocking could prove superior to COX-inhibitors.

However, regarding our findings in terms of IL-6 and PGE_2 secretion, it has to be mentioned that the possibility of contaminating cells contributing to the high levels of both mediators

cannot be fully excluded. As Wong et al. could recently show, AT2 cells do, in contrast to AT1 cells, express relatively low levels of IL-6 and only contamination with macrophages led to results, which matched those of other observers (Wong et al., 2013). In their works, they compared FACS-driven isolation procedures to protocols for magnetic isolation, with the latter yielding higher numbers of undesired cell types. As we also used magnetic separation, minor contamination with macrophages could still be possible. Nevertheless, we cultivated the cells for 6 days before collecting the supernatants for subsequent measurements and we therefore observed transdifferentiated AT1-like cells. It seems feasible to presume that their cytokine-expression pattern could be more closely related to AT1 cells than that of AT2 cells and the observed levels corresponded in fact to the findings of Wong et al. concerning IL-6 levels.

It is also noteworthy that PGE_2 is known for exerting beneficial effect on less distal epithelium in the airways, especially in tracheal and bronchial epithelium, both in cats and in humans in terms of wound closure (Savla et al., 2001). Furthermore, as reviewed in detail by Vancheri et al. in 2004 (Vancheri et al., 2004), PGE_2 is known for its protective effects in inflammatory conditions in the lung in contrast to other tissues. It is therefore surprising to find such clear evidence of a rather harmful event caused by the very same agent. However it would be necessary to carry out further experiments in *in-vivo* settings to elucidate the meaning and relevance of this decrease in electrical resistance, as, as it was previously mentioned, the protective effects of PGE_2 *in-vivo* are significant.

In conclusion, we could show here the importance of PGE_2 in the regulation of alveolar epithelial barrier function, and although the epithelial barrier disrupting effect of PGE_2 was partly unexpected, it indicates future therapeutic options for diseases such as ARDS. This is even more eminent, as pharmaceutical treatments are limited under these conditions and current algorithms focus on ventilation strategies. EP2 antagonists could therefore become a novel agent, as it seems likely that increase of PGE_2 levels might abolish the epithelial cell barrier via activating EP2 receptor, while barrier enhancing effect is expected on the endothelial cell layer which is then mediated by EP4 receptor (Konya et al., 2012; Su et al., 2013). Thus, it would seem beneficial, to selectively block the responsible EP2 receptor (at

least in mice) without interfering with the endogenous PGE_2 secretion and its beneficial effects on the endothelium. In contrast, COX-inhibitors (which were for example used by Su et al. to effectively suppress the barrier disrupting effect of bile acid) would most likely also abolish the endothelial barrier enhancement. However, *in vivo* experiments would be required to further confirm these assumptions.

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