# Aus dem Institut für Immunologie

# der Medizinischen Hochschule Hannover


## CHARACTERISATION OF PATHWAYS INVOLVED IN BACTERIAL OR VIRAL INDUCED EXACERBATIONS OF AIRWAY DISEASES

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## Contents

## Summary of the thesis in German

Samuel Mang; CHARACTERISATION OF PATHWAYS INVOLVED IN BACTERIAL OR VIRAL INDUCED EXACERBATIONS OF AIRWAY DISEASES

Die hier vorgelegte Arbeit ist im Rahmen meiner Doktorarbeit bei der Boehringer Ingelheim GmbH & Co. KG in Zusammenarbeit mit dem Institut für Immunologie der Medizinischen Hochschule Hannover angefertigt worden. Das Hauptaugenmerk der Arbeiten und insbesondere der Veröffentlichungen war es in einer Reihe von *in vitro* und *in vivo* Modellen, die Interaktion von Epithelzellen und Makrophagen zu untersuchen welche Teilaspekte der Idiopathischen Pulmonalen Fibrose (IPF) und der Chronisch Obstruktiven Lungenerkrankung (COPD) widerspiegeln.

Die IPF ist charakterisiert durch eine schrittweise, fokale, honigwaben-artige Fibrosierung der distalen Lunge. Die COPD ist charakterisiert durch eine chronische Inflammation und/oder emphysematöse Veränderungen der Lunge, bei der es zu Symptomen wie Auswurf, Husten und Atemnot kommen kann. Ein wichtiger Faktor in der Pathogenese der COPD ist Zigarettenrauch. Aber auch Partikel, Überreste von verbranntem Holz oder Kohle, Stäube oder Smog stehen im Verdacht die Krankheit auszulösen oder zumindest deren Progression zu beeinflussen. Der Verlauf der Krankheit wird bei beiden Erkrankungen durch akute Exazerbationen negativ beeinflusst. Diese Exazerbationen führen zu einer rapiden Verschlechterung der Lungenfunktion und erhöhen dadurch maßgeblich die Mortalität der Krankheit. Diverse Faktoren sind bisher mit Exazerbationen assoziiert worden. Diese Faktoren sind für die IPF zum größten Teil unbekannt, allerdings stehen z.B. Micro-Verletzungen des Lungengewebes, Viren wie Herpesviren und auch Inflammation im Verdacht Exazerbationen der IPF zu fördern. Im Falle der COPD werden bestimmte Viren wie Influenza (H1N1), Respiratory Syncytial

Virus (RSV) oder Bakterien wie non-typeable *Haemophilus Influenzae* (NTHi) oder *Pseudomonas aeruginosa* mit akuten Exazerbationen assoziiert.

Der Befall mit Bakterien oder Viren führt zu einer starken Entzündung (Bronchitis) der Atemwege und damit nicht nur zu einem unvorteilhaften Milieu für den Gasaustausch, sondern, er kann auch zu strukturellen Veränderungen in der Lunge beitragen (emphysematöse Veränderungen).

In der hier vorgelegten ersten Publikation mit dem Titel „Neutralization of both IL-1α/IL-1β plays a major role in suppressing combined cigarette smoke/virus-induced pulmonary inflammation in mice“ publiziert in Magazin „Pulmonary Pharmacology & Therapeutics“ (Bucher et al., 2017) wurde der Einfluss von Rauch und H1N1 auf murine Lungen und primäre humane bronchial Epithelzellen (HBEC) in Air-Liquid Interface (ALI) untersucht. In der zweiten Publikation mit dem Titel “Importance of the IL-1 axis in *Haemophilus influenzae* stimulated $M_1$ macrophages driving transepithelial signaling“ publiziert im Magazin „American Journal of Respiratory Cell and Molecular Biology“ (Mang et al., 2018) wurden die Interaktionen von primären humanen Small Airway Epithelzellen (SAEC) in Co-kultur mit GM-CSF ($M_1$) und M-CSF ($M_2$) vorstimulierten Makrophagen untersucht. Diese Co-kulturen wurden mit NTHi stimuliert. Beide Publikationen zeigten, dass sowohl der Virus als auch das Bakterium einen signifikanten Effekt auf das Epithel und auf die Immunzellen der Atemwege haben. Dieser Effekt der sich unter anderem als permeabilitätserhöhend zeigte, konnte durch Blockade des Interleukin 1 (IL-1) Signalweges beeinflusst werden. Es konnte in den zwei Veröffentlichungen gezeigt werden, dass im Virus Model vor allem IL-1α eine dominante Rolle spielt und im Model der bakteriell induzierten Permeabilität des Epithels, IL-1β die dominante Rolle spielt. Dabei ist aber zu beobachten, dass in beiden Fällen, das Entfernen beider Zytokine mit Hilfe von Antikörpern einen überadditiven Effekt hat. In der dritten Publikation mit dem Titel „Opposing effects of *in vitro* differentiated

macrophages sub-type on epithelial wound healing" publiziert im Journal „PLOS ONE" (Gindele & Mang et al., 2017) wurden die Interaktionen von verschiedenen Makrophagen Subtypen mit dem mechanisch geschädigten Epithel untersucht. Dabei wurde das SAEC-Epithel mit einer durch die Kultur gezogenen Verletzung versehen und verschieden stimulierte Makrophagen auf die Kultur gegeben. Hierbei konnte gezeigt werden, dass die Subtypen unterschiedliche Auswirkungen auf die „Wundheilung" des Epithels haben. Dabei war zu sehen, dass zu Beginn der „Wundheilung" eine Dedifferenzierung der Epithelzellen stattfand, was von einer schnellen Migration der Zellen zur Wunde hin und Proliferation hinter der Wundgrenze begleitet wurde. Dedifferenzierung der Epithelzellen wurde für $M_1$ und $M_2$ Makrophagen festgestellt. Die Fokal Adhesion Kinase (FAK) wurde herauf- und das Mucin Muc5AC heruntergesetzt durch $M_1$ im Gegensatz zu $M_2$. Dies legt nahe, dass $M_1$ Makrophagen die Motilität und die Epitheliale Mesenchymale Transition (EMT) verstärken.

Zusammenfassend ist die Aussage der drei Publikationen, dass Makrophagen bei der Wundheilung und bei der Reaktion auf bakterielle/virale Infektionen eine große Rolle spielen. Im Falle der Infektionen war dies primär durch den IL-1 Signalweg moduliert.

## Summary of the thesis

Samuel Mang; CHARACTERISATION OF PATHWAYS INVOLVED IN BACTERIAL OR VIRAL INDUCED EXACERBATIONS OF AIRWAY DISEASES

The work presented here is in support of my PhD thesis realized at Boehringer Ingelheim GmbH & Co. KG in collaboration with the Institute of Immunology of the Hannover Medical School. The main focus of the work, and in particular of the publications, was to investigate the importance of the pulmonary epithelium and lung macrophages in response to pathogenic stimuli or mechanical injury using a series of *in vitro* and *in vivo* models which resemble some aspects of Idiopathic Pulmonary Fibrosis (IPF) and Chronic Obstructive Pulmonary Disease (COPD).

IPF is characterized by a gradual, focal, honeycomb-like fibrosing of the distal lung that has been associated with micro injuries. COPD is characterized by chronic inflammation and/or emphysematous changes in the lung, where symptoms include excess sputum production, coughing, and respiratory distress. Cigarette smoke is suspected to be a major contributor to COPD pathophysiology, but also ambivalent particles, biomass, dusts and smog may contribute to or at least influence the disease progression. The course of the disease is negatively influenced in both diseases by acute exacerbations. These exacerbations lead to a rapid deterioration of lung function and thus significantly increase the mortality of the disease. Various factors have been associated with exacerbations. These factors are largely unknown for IPF but micro injuries, viruses like herpes virus and inflammation have been associated with exacerbations of IPF. In the case of COPD, certain viruses such as influenza (H1N1), Respiratory Syncytial Virus (RSV) or bacteria such as non-typeable *Haemophilus influenza* (NTHi) or *Pseudomonas aeruginosa* are suspected to trigger exacerbations. The infection

results in severe respiratory inflammation (bronchitis) and can lead to an unfavourable milieu for gas exchange but can also contribute towards structural changes (emphysematous changes).

In the first publication published in the journal „Pulmonary Pharmacology & Therapeutics“ (Bucher et al., 2017), entitled 'Neutralization of both IL-1α/IL-1β plays a major role in suppressing combined cigarette smoke/virus-induced pulmonary inflammation in mice', exposure to cigarette smoke and H1N1 was examined for effects in the lungs of mice and on primary human bronchial epithelial cells (HBEC) in Air-Liquid Interface (ALI). In the second publication entitled 'Importance of the IL-1 axis in *Haemophilus influenzae* stimulated $M_1$ macrophages driving transepithelial signaling', published in the journal „American Journal of Respiratory Cell and Molecular Biology“ (Mang et al., 2018), interactions of primary human small airway epithelial cells (SAEC) in co-culture with GM-CSF ($M_1$) and M-CSF ($M_2$) primed macrophages were determined. These co-cultures were stimulated with NTHi. Both publications show that both virus and bacteria have significant effects on the epithelium and on immune cells of the respiratory tract. This effect, which was demonstrated as a permeability increase, could be completely diminished by blocking interleukin 1 (IL -1) signalling. In these two publications we were able to show that IL-1α plays a dominant role in the virus model and IL-1β plays the dominant role in the bacterial induced permeability model. Furthermore, it can be observed that in both cases the removal of cytokines with antibodies has an over additive effect. In the third publication published in the journal „PLOS ONE“ (Gindele & Mang et al., 2017) entitled 'Opposing effects of *in vitro* differentiated macrophages sub-type on epithelial wound healing', the interactions of various macrophage subtypes with the mechanically damaged epithelium were examined. The SAEC epithelium was injured by introducing a lesion to the SAEC culture and different *in vitro* differentiated macrophage subtypes were added apically. It was found that the subtypes have different effects on the epithelial response to injury and repair. In the beginning of the repair process it was observed that the epithelial cells

dedifferentiated quickly, accompanied by rapid migration of cells to the wound edge and proliferation behind the wound edge. Dedifferentiation was reinforced by $M_1$ and $M_2$ macrophages but expression of Focal Adhesion Kinase (FAK) was enhanced and Muc5AC expression was lowered by $M_1$ compared to $M_2$ macrophages suggesting enhancement of motility and epithelial to mesenchymal transition (EMT).

In summary, the three publications show that during both wound healing and in response to bacterial and viral infection macrophage sub-types differentially impact the response of the epithelium. In the case of infection, this was modulated primarily via the IL-1 pathway.

## Introduction to the thesis

### Chronic Obstructive Pulmonary Disease (COPD)

Chronic Obstructive Pulmonary Disease (COPD) is an inflammation-driven pulmonary disease for which smoking is one of the important pathogenic factors. COPD is associated with lung function decline that is often accelerated by exacerbations. High exacerbation frequency has been associated with chronic systemic inflammation and mortality in patients with COPD (Agusti et al., 2012) and the inflammation and exacerbations are associated with influx of immune cells like neutrophils and macrophages into the lung (O'Donnell et al., 2006). The factors that are associated with COPD exacerbations include pathogens such as non-typeable *Haemophilus influenzae* (NTHi) (Bafadhel et al., 2015) or infections of the respiratory tract with viruses like Influenza (H1N1) which contribute to health status decline, disease progression and mortality (Wedzicha, 2015).

### Idiopathic Pulmonary Fibrosis (IPF)

Idiopathic pulmonary fibrosis (IPF) is defined as a specific form of chronic, progressive fibrosing interstitial pneumonia of unknown cause. It`s a form of idiopathic interstitial pneumonias, characterized by a progressive fibrotic remodelling of lung tissue and a steady deterioration of lung function parameters and dyspnoea (Raghu et al., 2011, Löh et al., 2017). IPF has been associated with various factors that influence the pathogenesis of the disease, one of which is repeated (micro-) injuries to the epithelium (Chambers and Mercer, 2015).

### Epithelial Injury

Epithelial injury in the lung can have several causes, including inflammation or mechanical injury induced for example by high peak pressure of ventilators. Such injuries must be repaired to ensure proper function of the epithelium as a barrier and to ensure proper gas exchange.

Inappropriate repair responses to macro- and micro-pulmonary epithelial injury have been linked to perturbation of epithelial barrier function and airway remodelling. It is possible that this is a consequence of epithelial-mesenchymal transition, in a number of respiratory diseases, including COPD (Wang et al., 2013) and IPF (Chapman, 2011). While the exact process of epithelial regeneration remains elusive it is believed that cells at the wound edge de-differentiate and flatten, enabling migration over the damaged area. The epithelial basal cells then proliferate and the wound is provisionally sealed with undifferentiated cells (Erjefalt et al., 1995). Subsequently, it is believed that a process of re-differentiation takes place and extracellular matrix proteins such as fibronectin are produced, which form the scaffold for the regenerated and fully differentiated epithelium (Erjefalt et al., 1997, Persson, 1996, Erjefalt et al., 1994). If this process is inappropriately executed non- or dysfunctional re-epithelialisation may take place. These structural changes are located in the lumen of the lower airways where there are macrophages present that have a great impact on the re-epithelialisation and structural repair mechanisms (Wynn and Vannella, 2016).

**Macrophage Subtypes**

Macrophage classification traditionally describes Granulocyte/Macrophage-Colony Stimulating Factor (GM-CSF; CSF2)-induced $M_1$-like and Macrophage-Colony Stimulating Factor (M-CSF; CSF1)-induced $M_2$-like subtypes (Martinez and Gordon, 2014). However, it has been suggested that the microenvironment and external factors lead to the existence of a more heterogeneous population of macrophage phenotypes (Xue et al., 2014).

In IPF, a number of groups have described lung macrophages as possessing $M_2$-associated markers, such as CD163 and it has been discussed that such macrophages are associated with a tissue remodelling phenotype. In contrast, COPD exacerbations (Barnes, 2004) have been shown to elicit an inflammatory response and promote an $M_1$ phenotype. Indeed, granulocyte-

macrophage colony stimulating factor (GM-CSF), $M_1$ macrophage differentiation factor, and product of cells activated during inflammation, is elevated during COPD (Ushach and Zlotnik, 2016, Day et al., 2014).

Further studies show that monocyte-derived macrophages (MDMs) from COPD patients produce more pro-inflammatory cytokines upon stimulation (Day et al., 2013) and airway macrophages express the GM-CSF dependent marker PPARγ (Schneider et al., 2014) which is lower in COPD and downregulated during COPD exacerbations (Zhao, 2011). Again, showing plasticity of these cells in a disease dependent context.

A direct interaction of a pro-inflammatory type of macrophage and the epithelium has been demonstrated. A study by Herold et al. demonstrated that resident alveolar macrophages disrupted the epithelial barrier through IL-1β leading to influx of exudate-macrophages into the lungs of mice (Herold et al., 2011). Interestingly, despite the inflammatory aspect and the use of steroids as a standard of care, COPD has been reported to be steroid insensitive (Barnes, 2004).

**Inflammatory Mediators**

Members of the IL-1 family such as IL-1α and IL-1β are pro-inflammatory cytokines, commonly useful for the initiation or maintenance of a response towards deleterious events like a bacterial infection. IL-1β is produced in a NACHT, LRR and PYD domains-containing protein 3 (NALP3) inflammasome / Caspase 1 dependent manner, whereas secreted IL-1α is produced via cleavage of the pro-peptide by calpain (Gabay et al., 2010). These cytokines bind to their primary receptor IL-1R1 and signal through downstream signaling mediators like p38-MAPK. Interestingly, these mediators are found to be increased in COPD patients and have been associated with chronic inflammation (Botelho et al., 2011, Pauwels et al., 2011, Rogliani et al., 2015).

Upon activation, inflammatory cells can secrete a range of potent pro-inflammatory cytokines amongst these are the aforementioned IL-1β and IL-1α, IL-6 and TNFα, chemokines such as MCP-1, metalloproteases, and reactive oxygen species (ROS) (Garlanda et al., 2013). The release of these inflammatory mediators might lead to endothelial cell activation, amplified inflammation, and to impaired epithelial barrier function. These effects have the potential to increase the susceptibility to viral/bacterial infections and subsequent exacerbations (Agusti et al., 2012, Hurst et al., 2006, Samara et al., 2010).

Clinical evaluation of p38-MAPK inhibitors, a kinase involved in pro-inflammatory signalling pathways, showed reduced neutrophil counts in sputum in an LPS challenge model (Singh et al., 2015) and improved lung function in COPD patients (MacNee et al., 2013). Recent evidence suggests that secreted IL-1 by alveolar macrophages may be the initiation factor of the inflammatory response to gram-negative pneumonia (Herold et al., 2011). Additionally, administration of Anakinra, the IL-1 signaling inhibitor IL-1RA reduced inflammation in other inflammatory diseases (Gibbs et al., 2005).

Taken together the considerations about epithelial injury, IL-1 signalling and general macrophage/epithelial interactions show their importance in lung disease biology. The established models can help to give insight into the biology of epithelial wound repair and COPD exacerbations. Furthermore, they might help to develop inhibitors that can target important pathways and find new drugs that can benefit patients that suffer from these severe diseases.

## Results

The research conducted during this dissertation lead to a number of findings and results that helped to advance current knowledge as shown in a number of publications. These publications are shown in the following section.

Commonly my contributions to these publications are the de-novo development of the Human Small Airway Epithelial Cell (SAECs) cultures, Monocyte derived macrophage subtype cultures and the experiments with these and similar cultures like Human Bronchial Epithelial Cells (HBECs).

**Publication #1: Neutralization of both IL-1α/IL-1β plays a major role in suppressing combined cigarette smoke/virus-induced pulmonary inflammation in mice**

For this publication I provided experimental *in vitro* knowledge, conducted experiments and did the data analysis for the *in vitro* DHBE (Diseased Human Bronchial Epithelial Cells) study. To conclude the data we had gotten from the animal experiments, we wanted to test the impact of the various antibodies on H1N1 infected, diseased (COPD Donor) Human Bronchial Epithelial Cells. To achieve this, I grew the cells into a differentiated monolayer, infected them with H1N1 and applied antibodies to the basolateral side. As a readout I measured single point Trans-Epithelial Resistance as a measure of permeability. Inflammatory cytokine concentrations were measured on the basolateral side via MSD. With these experiments, we were able to confirm effects the antibodies had shown in the *in vivo* setting and thus were able to demonstrate similar effects in human cells and in mice under these experimental conditions.

The findings in this paper had major implications on the hypothesis generation for the publication about effects of $M_{1.1}$ macrophage derived IL-1 on the epithelium.

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# Neutralization of both IL-1α/IL-1β plays a major role in suppressing combined cigarette smoke/virus-induced pulmonary inflammation in mice

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ABSTRACT

Smoking is an important risk factor for the development of chronic obstructive pulmonary disease (COPD) and viral infections are believed to be major triggers of exacerbations, which periodically lead to a worsening of symptoms. The pro-inflammatory IL-1 family members IL-1α and IL-1β are increased in COPD patients and might contribute to disease pathology. We investigated whether individual or combined inhibition of these cytokines reduced lung inflammation in cigarette smoke (CS)-exposed and H1N1-infected BALB/c mice. Animals were treated with individual or combined antibodies (Abs) directed against IL-1α, IL-1β or IL-1R1. Cells in BAL fluid and cytokines/chemokines in lung homogenate were determined. The viral load was investigated. Blocking IL-1α had significant suppressive effects on total cells, neutrophils, and macrophages. Furthermore, it reduced KC levels significantly. Blocking of IL-1β did not provide significant activity. In primary human bronchial epithelial air-liquid-interface cell cultures infected with H1N1, IL-1α Abs but not IL-1β Abs reduced levels of TNF-α and IL-6. Concomitant usage of Abs against IL-1α/IL-1β revealed strong effects *in vivo* and reduced total cells, neutrophils and macrophages. Additionally, levels of KC, IL-6, TNF-α, MCP-1, MIP-1α and MIP-1β were significantly reduced and ICAM-1 and MUC5 A/C mRNA expression was attenuated. The viral load decreased significantly upon combined IL-1α/IL-1β Ab treatment. Blocking the IL-1R1 provided significant effects on total cells, neutrophils and macrophages but was inferior compared to inhibiting both its soluble ligands IL-1α/IL-1β. Our results suggest that combined inhibition of IL-1α/IL-1β might be beneficial to reduce CS/H1N1-induced airway inflammation. Moreover, combined targeting of both IL-1α/IL-1β might be more efficient compared to individual neutralization IL-1α or IL-1β or inhibition of the IL-1R1.



## 1. Introduction

Smoking is a central risk factor for the development of COPD and viral infections of the respiratory tract are believed to be important triggers of exacerbations which contribute to a worsened health status, disease progression and mortality [1]. High exacerbation frequency has been associated with chronic systemic inflammation and mortality in patients with COPD [2].

Members of the IL-1 family such as IL-1α and IL-1β are pro-inflammatory cytokines that are found increased in COPD patients and might contribute to the detrimental chronic inflammation [3–5]. Via binding to their primary receptor IL-1R1, these cytokines have the ability to recruit and/or to activate a variety of immune cells and immunocompetent cells, such as macrophages, endothelial cells, neutrophils and epithelial cells. Upon activation, these cells can secrete a range of potent pro-inflammatory cytokines such as IL-6 and TNF-α, chemokines such as MCP-1, MIP-1α and MIP-1β, metalloproteases, and reactive oxygen species (ROS) [6]. The release of these inflammatory mediators might lead to further endothelial cell activation, amplified inflammation, and to

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**Abbreviations**

| | |
|---|---|
| Ab | Antibody |
| ALI | Air-liquid-interface |
| BAL | Bronchoalveolar Lavage |
| CS | Cigarette smoke |
| ERK | Extracellular Signal-Regulated Kinases |
| H1N1 | Influenza A/PR/8/34 |
| IL | Interleukin |
| i.p. | Intraperitoneal |
| i.t. | Intratracheal |
| IU | Infectious Units |
| MUC | Mucin |
| TER | Transepithelial Electrical Resistance |

impaired epithelial barrier function potentially increasing the susceptibility to viral/bacterial infections and subsequent exacerbations [2,7,8].

Pre-clinical models employing cigarette smoke (CS)-exposure are widely used to mimic aspects of airway inflammation [9,10] and have been used previously to link the IL-1 pathway to CS-induced airway inflammation [3,4,11]. Increased IL-1α and IL-1β levels have been detected separately in CS-treated animals and inhibition of the respective signaling pathways resulted in anti-inflammatory activity. For example, neutralization/blockade of IL-1α or IL-1R1 by antibodies (Abs) or genetic depletion of IL-1α, IL-1β or IL-1R1 has been reported to reduce CS-induced inflammation [3,4,11,12].

Notably, recent clinical Phase II studies using the IL-1β Ab Canakinumab as well as the IL-1R1 Ab MEDI-8968 did not meet their primary endpoints ($FEV_1$, exacerbation rate) in patients with COPD [5,13]. Consequently, it remains an open question whether targeting the IL-1 pathway could be a beneficial therapeutic option for the treatment of COPD.

In the current work, the individual or combined contribution of IL-1α and IL-1β and the role of IL-1R1 were investigated for the first time head-to-head in a recently described murine model where inflammation is triggered by CS and H1N1 mimicking aspects of an exacerbated inflammatory COPD phenotype [14]. We found that in particular combined treatment with IL-1α and IL-1β Abs strongly reduced inflammation and might be an attractive option to reduce inflammation and exacerbations in COPD patients.

## 2. Material & methods

### 2.1. Animals

Female BALB/cAnNCrl mice, age of 8–12 weeks, were purchased from Charles River, Germany. The animals received water and food *ad libitum* and were housed in a specific pathogen-free facility at 20–25 °C. All experimental procedures were performed in accordance with European and local animal welfare regulations (Regierungspräsidium Tübingen, Germany, approved animal experimental licenses TVV 12-014 and 14-016).

### 2.2. Cigarette smoke-exposure and infection

CS-exposure and virus infection was performed as described before [14,15]. In brief, mice were exposed to CS of four cigarettes daily (Roth-Händle without filters, Badische Tabakmanufaktur Roth-Händle, Germany) inside a perspex box for nine days. Five days prior to readout, mice were anaesthetized with 3% isoflurane and infected two hours after CS-exposure by administering 30 infectious units (IU) influenza virus A/PR/8/34 (H1N1) diluted in 50 μl PBS intranasally. H1N1 was provided by Boehringer Ingelheim, Canada.

### 2.3. Antibodies

Abs against mouse IL-1α (clone ALF-161, hIgG, eBioscience, USA) and mouse IL-1R1 (clone JAMA-147, hIgG, BioXCell, USA) were sourced commercially, as well as the isotype control Ab eBio299Arm (hIgG, eBioscience, USA). Anti-mouse IL-1β Ab #315B4G5 (mIgG1) was developed in-house; MOPC-21 (mIgG1, BioXCell, USA) was used as isotype control. Further information on generation and *in vitro* characterization of the anti-mouse Abs can be found in the supplemental data. In brief, all Abs were tested for endotoxin content (Endosafe-PTS, Charles River, USA), aggregation (Äkta chromatography, GE Healthcare, United Kingdom) and cellular potency (Supplemental Fig. S1). All Abs contained <5 EU/mg endotoxin and were >95% monomeric.

In the *in vitro* studies using human primary epithelial cells, IL-1α was neutralized by 100 nM of clone #4414 (mIgG2A, R&D Systems, USA; isotype control clone #20102, R&D Systems, USA) or IL-1β by 100 nM Canakinumab (hIgG1, Komptur Apotheke, Germany; isotype control anti-TNP, Boehringer Ingelheim Pharma Gmbh & Co KG), i.e., at concentrations reaching maximal efficacies in cellular assays (data not shown).

In some studies, the anti-mouse IL-18 Ab #63E1C6 was tested. The generation and characterization of this Ab is described in the Supplemental Fig. S2.

### 2.4. Target engagement models

Target engagement models were developed using i.t. application of recombinant mouse IL-1α (#14-8011, eBioscience, USA) or IL-1β (#200-01B, Peprotech, Germany), respectively, to determine the efficacious dose for each Ab for subsequent experiments. In brief, dose-range finding and kinetic studies suggested 10 ng recombinant IL-1α or 10 ng recombinant IL-1β administered i.t. to induce submaximal phosphorylation of ERK in the lungs, peaking 20 min after administration (data not shown). Subsequently, the IL-1α Ab, the IL-1β Ab or the IL-1R1 Ab, respectively, was administered i.p. 18 h before the i.t. stimulus. 20 min after stimulation with the recombinant ligand, the animals were sacrificed; the lungs were removed and subjected for homogenization. The ratio pERK/total ERK was determined according to the instructions of the manufacturer (#K151DWD, Meso Scale Diagnostics, USA).

### 2.5. Antibody treatment in CS/H1N1 model

Mice were treated i.p. with Abs directed against IL-1α (200 μg/mouse) and/or IL-1β (300 μg/mouse) or with Abs antagonizing the IL-1R1 (300 μg/mouse). Treatment was performed on day 1, 3, 5, 8, 10 of the experiment. Isotype controls were used to adjust the amount of protein per mouse. Notably, all mice received the same amount of Abs, either as anti-target Ab or as isotype control Ab or as combinations thereof.

### 2.6. Measurement of cell counts in BAL fluid

On the last day of the experiment, ~20 h after the last CS-exposure, mice were sacrificed by intraperitoneal injection of an overdose pentobarbital (Merial GmbH, Germany). Lungs were lavaged twice with 0.8 ml of lavage buffer (PBS containing 1% BSA). Cell counts in BAL fluid were measured and differentiated using a Sysmex XT-1800i automated haematology analyzer.

### 2.7. Cytokine measurement in lung homogenate

Lungs were removed and homogenized using a FastPrep-24 Sample Preparation System (MP Biomedicals, USA). Hank's Salt Solution containing 1% BSA, 0.1% 0.5 M EDTA, Protease and RNase-Inhibitor was used as homogenization buffer. Cytokine levels in lung homogenate were assessed using MSD multiplex technology (Meso Scale Diagnostics, USA) or sandwich ELISA (#DY400, R&D Systems, USA) according to the manufacturer's instructions.

### 2.8. Detection of H1N1, MUC5A/C and ICAM-1 expression

H1N1, MUC5A/C and ICAM-1 expression in lung homogenate were determined by qPCR. In brief, RNA was isolated and purified from 50 μl lung homogenate using the RNeasy Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) was used to generate cDNA from 1 μg of total RNA according to the manufacturer's instructions. qPCR was performed using Taqman technology. MUC5A/C and ICAM-1 gene expression assays were sourced from Life Technologies, USA. For detection of H1N1, the following primers and probe were obtained from Sigma Aldrich, Germany:

Forward: 5′-GATCAACTTCTGTCATCCAGCAA-3′
Reverse: 5′-TTCTGCACATCATAATTAGGAGTATCAAT-3′
Probe: 5′-CACCATCCAACGGAGCACAGGAGAT-3′.

The probe was labeled with the 5′ reporter dye 6-carboxyfluorescein (FAM) and the 3′ quencher dye 6-carboxytetramethylrhodamin (TAMRA). 2 μl of cDNA in a total volume of 10 μl with QuantiFast Probe PCR Kit (Qiagen, Germany) were used for Taqman. 18S rRNA (Life Technologies, USA) was assayed as internal control. Three technical replicates were generated. Additional reaction mixes containing 2 μl water instead of cDNA served as no template control.

### 2.9. Air-liquid-interface cell culture experiments

Diseased human bronchial epithelial cells (derived from a patient with COPD with a smoking history of over 40 years; Product code 00195275S; Lot nr. 0000332498) were obtained from Lonza, Germany. As stated by Lonza, the cells were isolated from donated human tissue after obtaining permission for their use in research applications by informed consent or legal authorization. Cells were thawed and cultured in a T75 culture flask overnight in expansion medium (PneumaCult-Ex basal medium containing 10% PneumaCult-Ex supplements, 1 μl/ml hydrocortisone, 10 μl/ml PenStrep). Cells were grown to a confluency of 80%. Subsequently, reagent pack subculture reagents (Lonza, Germany) were used for trypsination. Cells were counted and transferred into a falcon tube. 35.000 cells were added onto Transwell® Permeable Support filters (6.5 mm Insert, 0.4 μm polyester membrane, Costar, USA). Four days after addition of the cells onto the transwell filters, apical medium was removed and basal medium was switched to differentiation medium (Pneumacult-ALI basal medium containing 10% Pneumacult-ALI supplements, 1% Pneumacult-ALI maintenance supplements, 2 μl/ml 0.2% heparin sodium salt in PBS, 4 μl/ml hydrocortisone, 10 μl/ml PenStrep). Air-liquid-interface (ALI) cells were grown for three weeks and differentiated into basal cells, ciliated cells, and mucus-producing cells. Medium was changed every second or third day. Apical mucus was removed as required by washing with PBS. Hydrocortisone and PenStrep was removed from the medium five days prior to treatment and H1N1 infection. Anti-human IL-1α Ab (100 nM) or anti-human IL-1β Ab (100 nM) was added to the basal medium 6 h prior to infection with $1.5 \times 10^5$ IU H1N1 in 30 μl PBS which was added on the apical side of the cells. Appropriate isotypes were used as controls. Readout was performed 48 h post infection (p.i.). Cytokines were measured in supernatant using MSD multiplex technology as described in section 2.7. Transepithelial Electrical Resistance (TER) values were assessed in [Ohm] using an EVOM device (World Precision Instruments, USA) before treatment (t0) and after 48 h (t48) and normalized to $cm^2$ cell culture and finally reported as % change from t0 to t48.

### 2.10. Software and statistical analysis

Microsoft Office 2010 and GraphPad Prism 7.00 were used for data analysis and presentation. Multiple comparisons were performed by one-way ANOVA with Bonferroni's multiple comparisons post-test. The inhibitory activity of the treatments is additionally reported as % inhibition of positive control over baseline (negative control group). To achieve this, the mean of the negative control group was subtracted from all other values. The baseline-corrected values were then compared to the mean of the positive control group which was set to 100%.

Data is expressed as mean ± SEM. $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$ represents significant differences compared to the control.

## 3. Results

### 3.1. Abs against IL-1α, IL-1β and IL-1R1 show high target engagement in vivo

Mechanistic target engagement models were established to determine doses for each Ab that result in high target occupancy in the lung *in vivo*. The experimental scheme is illustrated in Fig. 1A. Administration of 10 ng recombinant mouse IL-1α or IL-1β resulted in significant phosphorylation of ERK (Fig. 1B and C). Treatment with the respective Abs 18 h prior to the i.t. stimulation with mouse IL-1α or IL-1β revealed strong suppressive effects on the phosphorylation of ERK when using ≥300 μg IL-1R1 Ab, ≥ 100 μg IL-1α Ab, or ≥300 μg IL-1β Ab.

### 3.2. Anti-IL-1α but not anti-IL-1β treatment reduces inflammatory cell numbers in BAL fluid and combined inhibition of IL-1α and IL-1β shows additive activity

In order to study the relevance of IL-1α or IL-1β in airway inflammation, a combined CS and H1N1 challenge model was used that was recently established and shows an aggravated inflammatory response after combined smoke and viral challenge [14]. Fig. 2A shows the experimental scheme.

Both IL-1α and IL-1β were strongly induced in lung homogenate of CS-exposed and H1N1-infected mice (Fig. 2B). Based on the target engagement results, CS/H1N1-exposed mice were treated with Abs against IL-1α (200 μg/mouse) or IL-1β (300 μg/mouse) or both. Neutralization of IL-1β alone failed to reduce the total cell, neutrophil, or macrophage numbers in BAL fluid significantly (Fig. 2C–E). In contrast, neutralization of IL-1α resulted in significantly reduced levels of total cells (52% inhibition), neutrophils (53% inhibition) and macrophages (52% inhibition). The strongest suppressive effects were achieved by combined neutralization of both IL-1α and IL-1β. Dual neutralization of the mediators resulted in additive activity and significantly reduced levels of total cells (71% inhibition), neutrophils (74% inhibition) and macrophages (73% inhibition).

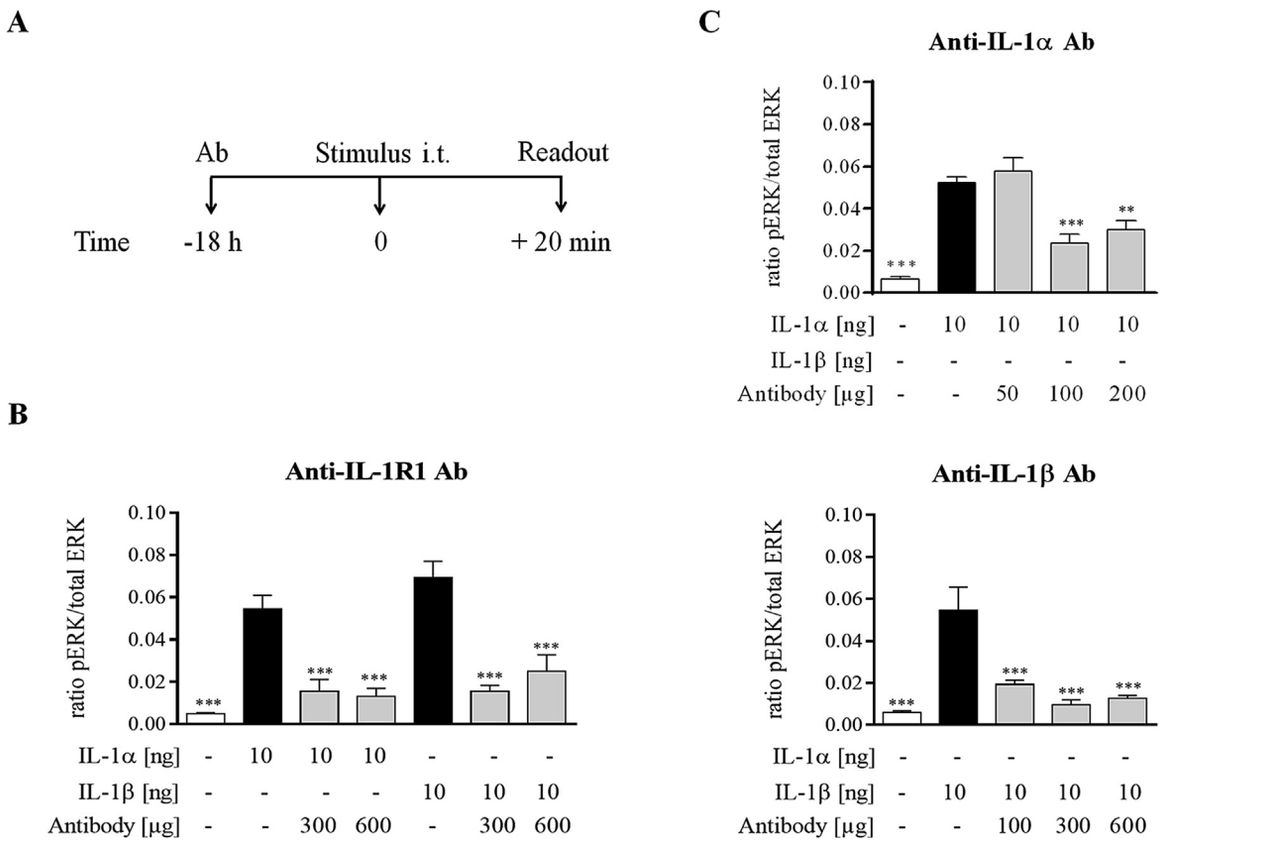


**Fig. 1.** Target engagement for IL-1α, IL-1β and IL-1R1 Abs. (A) Mice were treated i.p. with (B) IL-1R1 (#JAMA-147) antibodies (Abs) or (C) IL-1α (#ALF-161) or IL-1β (#315B4G5) Abs 18 h prior to i.t. administration of 10 ng recombinant mouse IL-1α or IL-1β. Amount of Ab per mouse was adjusted by administration of the corresponding isotype controls as described in material and methods. Phosphorylated ERK was measured in lung homogenate 20 min after administration of the stimulus. Mean values ± SEM of n = 4 mice are shown. ***p < 0.001, **p < 0.01 represent significant differences compared to the positive control group.

### *3.3. Combined inhibition of IL-1α and IL-1β strongly suppresses inflammatory mediators in lung homogenate of CS/H1N1 challenged mice*

In addition to BAL cell counts, pro-inflammatory cytokines and chemokines were measured in lung homogenate of CS/H1N1 challenged mice treated with Abs against IL-1α (200 µg/mouse) or IL-1β (300 µg/mouse) or with an Ab combination thereof. The results are displayed in Table 1. Administration of IL-1α Abs had significant suppressive activity on KC, administration of IL1-β Abs had significant suppressive activity on MIP-1β; but individual application of the Abs did not show further significant suppressive effects on the measured mediators. Interestingly, combined neutralization of IL-1α and IL-1β significantly reduced the expression levels of all six measured cytokines/chemokines (IL-6, KC, TNF-α, MCP-1, MIP-1α and MIP-1β), with effect sizes ranging from 51% inhibition (MIP-1α) up to full inhibition (KC). In summary, dual blockade of IL-1α and IL-1β was the most efficacious treatment, illustrating either additive (MCP-1, TNF-α, MIP-1β) or over-additive (KC, IL-6 MIP-1α) suppressive effects.

### *3.4. Anti-IL-1α but not anti-IL-1β treatment has anti-inflammatory activity in H1N1-infected air-liquid-interface cell culture*

In order to translate the *in vivo* findings to human conditions, we investigated the effects of IL-1α and/or IL-1β Ab treatment in primary human bronchial epithelial cells grown as an ALI cell culture system. Fig. 3A illustrates the experimental scheme. After a 35 day culture, the ALI culture consists of a differentiated epithelium containing basal cells, ciliated cells, and mucus-producing cells that was infected apically with heat-inactivated H1N1 or viable H1N1; though only the latter treatment resulted in a drop in TER values and a release of inflammatory mediators such as TNF-α and IL-6 (data not shown).

Having successfully established the ALI differentiation protocol and H1N1 infection *in vitro*, we subsequently studied the functional relevance of IL-1α and IL-1β in an ALI setting with cells derived from a patient with COPD and a >40 years smoking history. Upon infection, both IL-1α and IL-1β levels were induced, however, the expression of IL-1α was much stronger than IL-1β (Fig. 3B). Subsequently, cells were treated with antibodies against IL-1α or IL-1β or both and infected with H1N1.

In line with the *in vivo* studies, treatment with an IL-1α Ab but not with an IL-1β Ab had anti-inflammatory activity and reduced loss of TER (Fig. 3C) and levels of inflammatory TNF α and IL-6 (Fig. 3D and E), whilst isotype controls had no effect. In this setting, concomitant inhibition of IL-1α or IL-1β did not provide higher efficacy than targeting IL-1α alone.

### *3.5. Combined IL-1α/IL-1β neutralization is superior in reducing inflammatory cells and the loss of body-weight compared to blockade of IL-1R1 in CS/H1N1 challenged mice*

Next, we directly compared the anti-inflammatory effect of combined IL-1α/IL-1β Ab treatment on the CS/H1N1-driven inflammatory cell influx in BAL fluid to the effect of an antibody

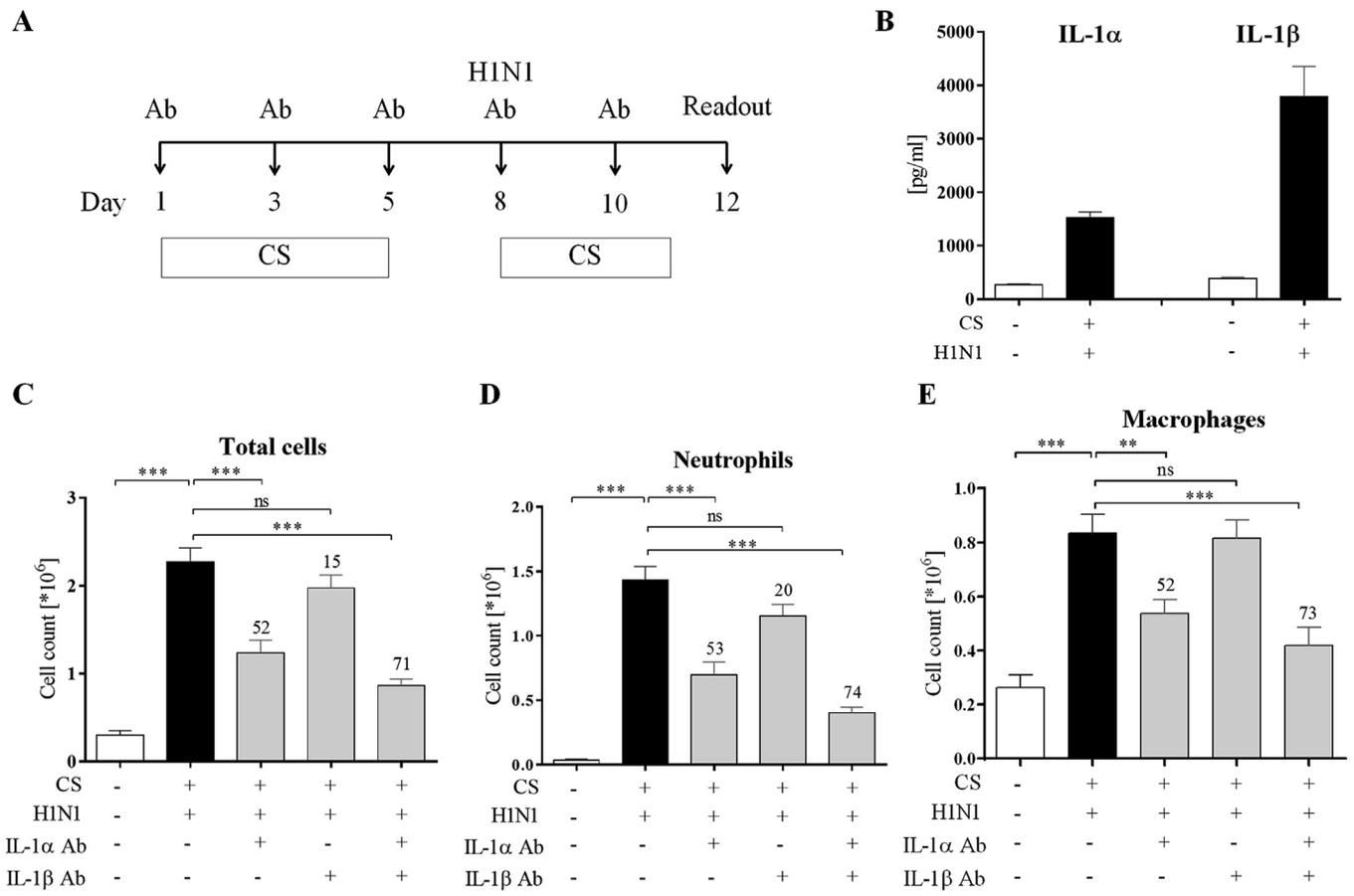


**Fig. 2.** Treatment of CS-exposed and H1N1-infected mice with IL-1α and/or IL-1β Abs. (A) Mice were exposed to cigarette smoke (CS) for a total of nine days and infected with influenza virus (H1N1) on day 8. Every second or third day, respective mice were treated with antibodies (Abs) targeting IL-1α or IL-1β or a combination thereof and adjusted with isotype control Abs. (B) At the end of the study, lungs were excised and homogenized, as detailed in material and methods. Levels of IL-1α and IL-1β in lung homogenate were measured using ELISA and MSD, respectively. (C) Total cells, (D) neutrophils and (E) macrophages in BAL fluid. Mean values ± SEM of n = 7–8 mice are shown. ***p < 0.001, **p < 0.01 represent significant differences compared to the CS/H1N1 group. The inhibitory activity of the treatments is reported as % inhibition of CS/H1N1 over baseline (negative control group).

**Table 1**
Cytokine levels in the lung homogenate of CS/H1N1-exposed mice after treatment with individual IL-1α or IL-1β antibodies or a combination of both antibodies. Mean values ± SEM of n = 7–8 mice are shown. ***p < 0.001, **p < 0.01, *p < 0.05 represent significant differences compared to the CS/H1N1 group. The inhibitory activity of the treatments is reported as % inhibition of CS/H1N1 over baseline (negative control group).

| Group | | Amount ± SEM [pg/ml] | % Inhibition [CS + H1N1] over baseline | Amount ± SEM [pg/ml] | % Inhibition [CS + H1N1] over baseline |
|---|---|---|---|---|---|
| | | KC | | IL-6 | |
| CS | | 192.0 ± 9.9 | | 22.3 ± 1.1 | |
| *CS + H1N1* | | *2371.0 ± 327.4* | | *4670.7 ± 845.7* | |
| Anti-IL- | 1α | 1363.0 ± 183.6 | 46.2** | 5355.0 ± 1274.5 | <0 |
| | 1β | 1800.0 ± 236.7 | 26.2 | 2960.6 ± 712.5 | 36.8 |
| | 1α + 1β | 181.3 ± 8.8 | 100*** | 1056.4 ± 105.0 | 77.8* |
| | | TNF-α | | MCP-1 | |
| CS | | 28.1 ± 1.2 | | 85.4 ± 4.8 | |
| *CS + H1N1* | | *277.0 ± 26.3* | | *3045.2 ± 457.4* | |
| Anti-IL- | 1α | 204.8 ± 24.0 | 29 | 2263.2 ± 312.1 | 26.4 |
| | 1β | 215.9 ± 34.8 | 24.6 | 2261.4 ± 377.0 | 26.5 |
| | 1α + 1β | 104.3 ± 6.4 | 69.4*** | 1371.6 ± 148.4 | 56.5** |
| | | MIP-1α | | MIP-1β | |
| CS | | 71.2 ± 3.1 | | 538.9 ± 17.2 | |
| *CS + H1N1* | | *472.1 ± 46.0* | | *6569.0 ± 905.9* | |
| Anti-IL- | 1α | 412.7 ± 29.5 | 14.8 | 4727.4 ± 690.6 | 30.5 |
| | 1β | 411.6 ± 38.8 | 15.1 | 4112.0 ± 750.9 | 40.7* |
| | 1α + 1β | 266.8 ± 9.9 | 51.2*** | 2043.0 ± 258.3 | 75.1*** |

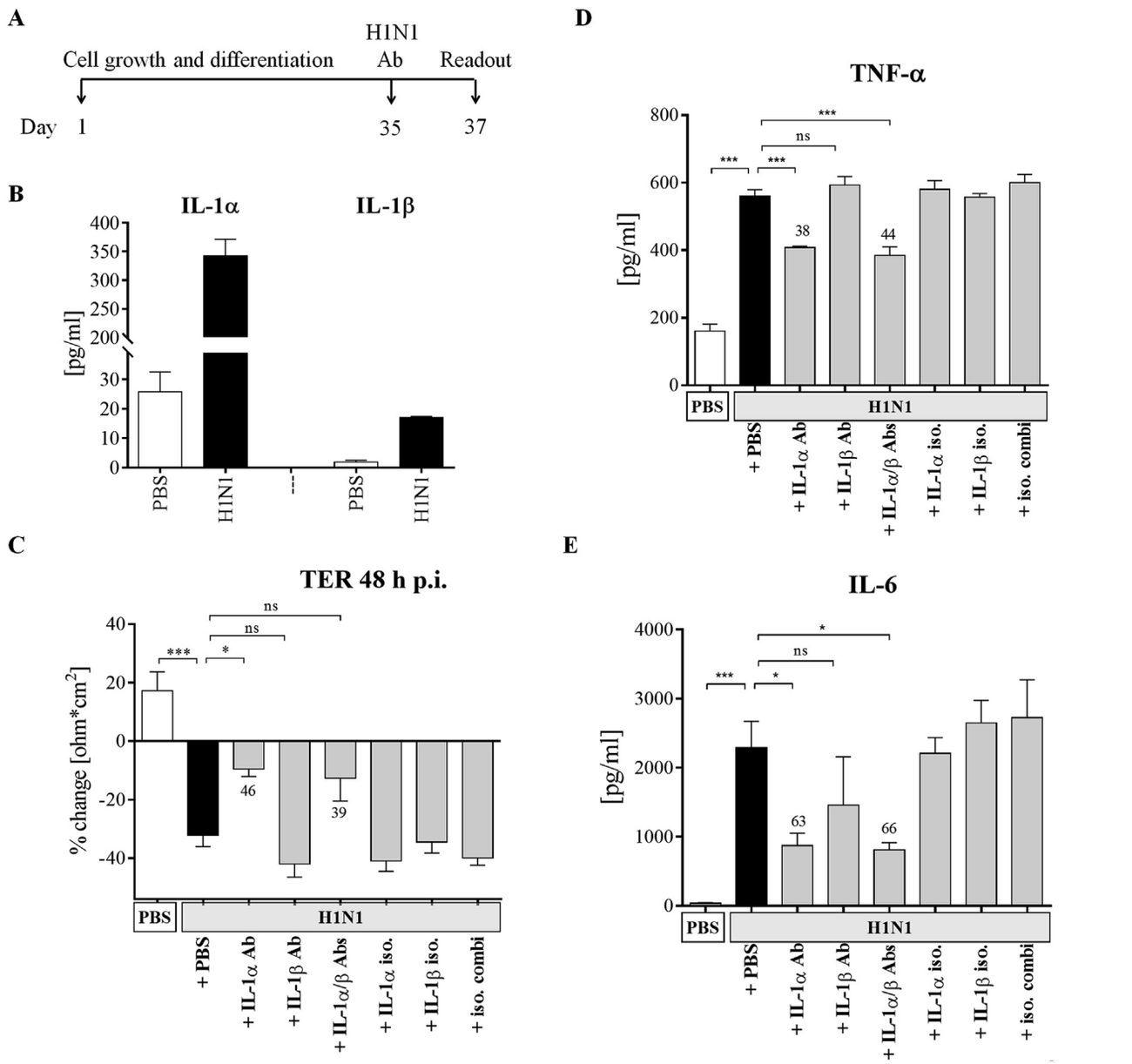


**Fig. 3.** Treatment of diseased primary human bronchial epithelial (DHBE) cells with IL-1α and/or IL-1β Abs or a combination thereof in an air-liquid-interface (ALI) setup. (A) Experimental setup for H1N1 infection. (B) Effects of H1N1 infection on basal IL-1α and IL-1β release. (C) Effects of IL-1α and/or IL-1β Abs or respective isotype ('iso.') controls on TER. Absolute TER values before H1N1 infection (t0) were 99.98 ohm*cm$^2$ and dropped to 72.7 ohm*cm$^2$ 48 h (t48) post infection (p.i.), whilst PBS-treated cells had an increased TER value of 117 ohm*cm$^2$ after 48 h. Reported is % change between t0 and t48 per treatment group. Effects of IL-1α and/or IL-1β Abs or respective isotype ('iso.') controls on TNF-α (D) and IL-6 (E). Mean values ± SEM of n = 4 wells are shown. ***p < 0.001, **p < 0.01, *p < 0.05 represent significant differences compared to the H1N1 group. Isotype controls were not significantly different from the H1N1 group. The inhibitory activity of the treatments is reported as % inhibition of H1N1+PBS over baseline (PBS).

targeting the IL-1R1 in our mouse model. The experimental scheme is shown in Fig. 4A. Again, IL-1α/IL-1β neutralization resulted in a strong and significant reduction of CS/H1N1-induced increase of inflammatory cells. Total cell numbers were reduced by 86%, neutrophil counts by 80% and macrophage counts by 95% (Fig. 4C–E). Blockade of the IL-1R1 decreased counts of total cells (62%), neutrophils (57%) and macrophages (72%) as well but the reduction of inflammatory cell counts was significantly less pronounced upon antagonism of IL-1R1 compared to combined neutralization of its soluble ligands IL-1α/IL-1β (Fig. 4C–E). The changes in inflammatory cells were also reflected by changes in the loss of body-weight (Fig. 4B). All treatments reduced the model-induced loss of body-weight significantly. As seen for inflammatory cells, combined neutralization of both IL-1α/IL-1β revealed a stronger effect compared to blockade of the IL-1R1.

### *3.6. Combined IL-1α/IL-1β neutralization is superior in reducing cytokine protein expression, viral load, and MUC5A/C and ICAM-1 mRNA expression in lung homogenate compared to blockade of IL-1R1 in CS/H1N1 challenged mice*

Table 2 illustrates cytokine/chemokine levels measured in lung homogenate of CS/H1N1 challenged mice after neutralization of IL-1α/IL-1β or blockade of IL-1R1. Treatment with the Ab combination exerted stronger effects than the IL-1R1 Ab and reduced levels of KC, IL-6, TNF-α, MCP-1, MIP-1α or MIP-1β significantly. Blockade of

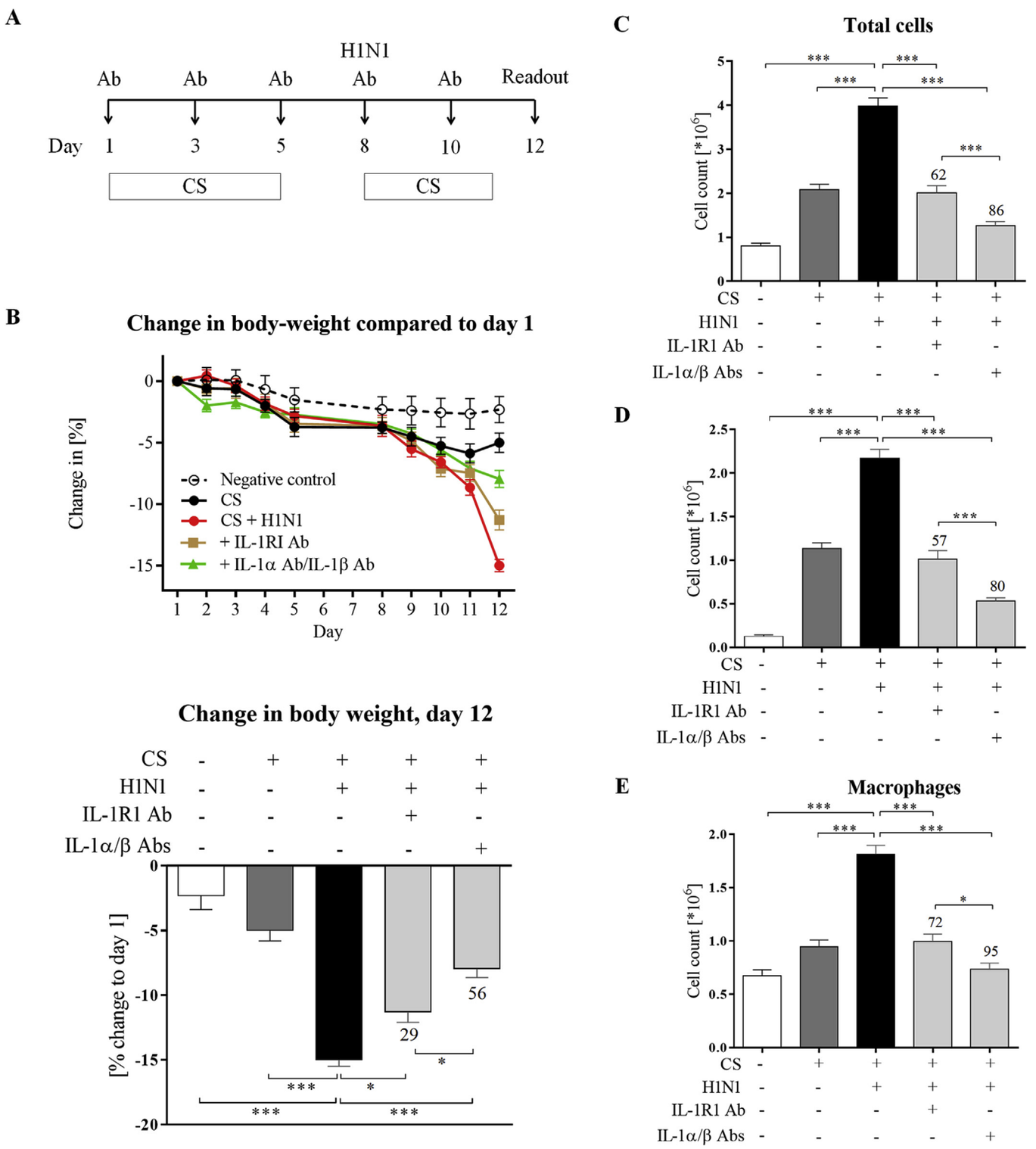


**Fig. 4.** Treatment of CS-exposed and H1N1-infected mice with IL-1R1 Ab or combined IL-1α/IL-1β Abs. (A) As described in the legend of Fig. 2, mice were exposed to cigarette smoke (CS) for a total of nine days and infected with influenza virus (H1N1) on day 8. Mice were treated with an antibody (Ab) targeting IL-1R1 or a combination of Abs targeting IL-1α/IL-1β and adjusted with isotype control Abs. (B) Body-weight loss is displayed as time course and as % change at day 12 compared to day 1. (C) Total cells, (D) neutrophils and (E) macrophages in BAL fluid are illustrated. Mean values ± SEM of n = 7–8 mice are shown. ***$p < 0.001$, *$p < 0.05$ represent significant differences compared to the CS/H1N1 group or the IL-1R1 group, respectively. The inhibitory activity of the treatments is reported as % inhibition of CS/H1N1 over baseline (negative control group).

**Table 2**
Cytokine levels in the lung homogenate of CS/H1N1-exposed mice after treatment with combined IL-1α/IL-1β Abs or IL-1R1 Abs. Mean values ± SEM of n = 7–8 mice are shown. ***p < 0.001, **p < 0.01 represent significant differences compared to the CS/H1N1 group. The inhibitory activity of the treatments is reported as % inhibition of CS/H1N1 over baseline (negative control group).

| Group | | Amount ± SEM [pg/ml] | % Inhibition [CS + H1N1] over baseline | Amount ± SEM [pg/ml] | % Inhibition [CS + H1N1] over baseline |
|---|---|---|---|---|---|
| | | KC | | IL-6 | |
| Neg. control | | 72.0 ± 5.2 | | 8.9 ± 0.6 | |
| CS | | 426.8 ± 26.3 | | 29.8 ± 1.8 | |
| *CS + H1N1* | | *3272.0 ± 215.5* | | *15549.0 ± 1341.0* | |
| Anti-IL- | 1R1 | 839.2 ± 131.2 | 76.0*** | 7299.8 ± 317.6 | 53.1*** |
| | 1α+1β | 275.4 ± 19.5 | 93.6*** | 4176.3 ± 282.6 | 73.2*** |
| | | TNF-α | | MCP-1 | |
| Neg. control | | 11.1 ± 0.5 | | 41.2 ± 2.0 | |
| CS | | 29.0 ± 0.8 | | 216.1 ± 17.2 | |
| *CS + H1N1* | | *416.3 ± 23.2* | | *4597.0 ± 344.0* | |
| Anti-IL- | 1R1 | 398.7 ± 21.4 | 4.3 | 4140.5 ± 201.0 | 10 |
| | 1α+1β | 268.9 ± 20.0 | 36.4*** | 2844.4 ± 328.9 | 38.5*** |
| | | MIP-1α | | MIP-1β | |
| Neg. control | | 1368.1 ± 161.5 | | 595.1 ± 70.0 | |
| CS | | 1221.8 ± 81.6 | | 854.7 ± 74.1 | |
| *CS + H1N1* | | *7449.0 ± 533.9* | | *19900.2 ± 1908.0* | |
| Anti-IL- | 1R1 | 7720.6 ± 613.6 | <0 | 16430.6 ± 1431.0 | 18 |
| | 1α+1β | 4750.2 ± 427.0 | 44.4*** | 12600.5 ± 1564.0 | 37.8** |

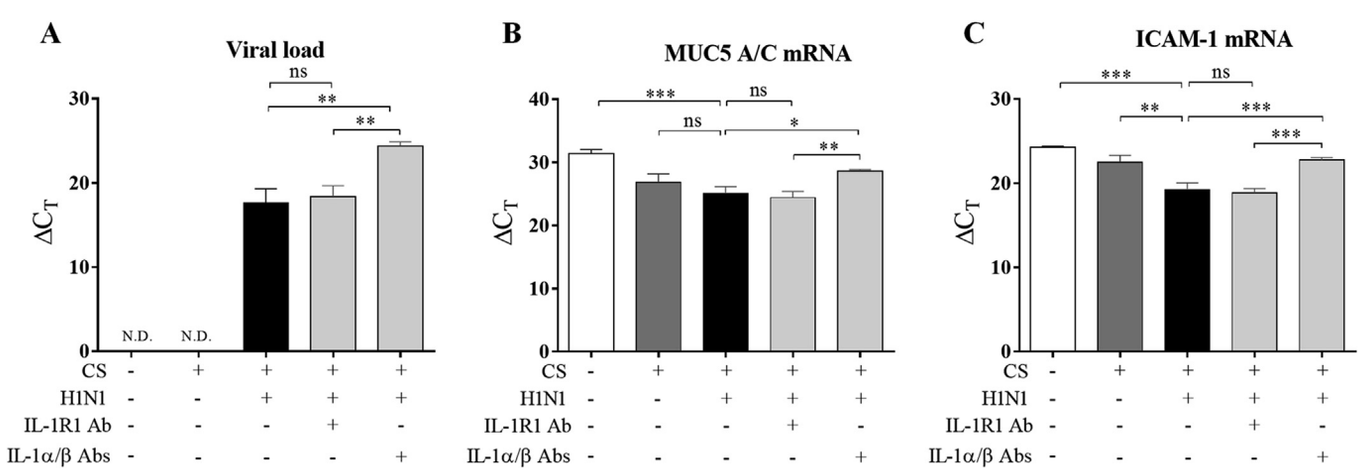


**Fig. 5.** Detection of H1N1 and gene expression analysis of ICAM-1 and MUC5A/C. Mice were exposed to cigarette smoke (CS) for a total of nine days and infected with influenza virus (H1N1) on day 8. Mice were treated with antibodies (Abs) as described before. qPCR was used for detection of (A) viral genomes and for gene expression analysis of (B) MUC5A/C and (C) ICAM-1 as described in material and methods. $\Delta C_T$-values are illustrated. Mean values ± SEM of n = 7–8 mice are shown. ***p < 0.001, **p < 0.01, *p < 0.05 represent significant differences compared to the CS/H1N1 group or the IL-1R1 group, respectively.

the IL-1R1 only resulted in reduced cytokine levels of KC and IL-6.

Beyond the effects on inflammatory mediators, we also investigated the effect of treatment of CS/H1N1-exposed mice with combined neutralization of IL-1α and IL-1β versus the blockade of IL-1R1 on the viral load using qPCR (Fig. 5A). In all cases, viral genomes were detectable in the infected animals. A significant reduced viral load was observed in the combined IL-1α/IL-1β Ab treated mice, indicated by a higher $\Delta C_T$-value of 24.4 compared to the control animals with a $\Delta C_T$-value of 17.6.

Furthermore, MUC5A/C and ICAM-1 mRNA expression in the lungs was investigated. Both, MUC5A/C and ICAM-1 mRNA were significantly elevated in CS/H1N1 challenged mice (Fig. 5B and C). Whilst the combined IL-1α/IL-1β Ab treatment showed significant effects to reduce MUC5A/C and ICAM-1 mRNA expression, treatment with the IL-1R1 Ab did not result in suppressive effects. In conclusion, combined treatment with the IL-1α/IL-1β Abs was significantly more efficacious than treatment with IL-1R1 Ab in reducing viral load and expression of MUC5A/C and ICAM-1.

## 4. Discussion

High exacerbation frequency has been associated with chronic systemic inflammation and mortality in patients with COPD [2]. The pro-inflammatory IL-1 family members IL-1α and IL-1β are increased in patients with stable COPD and during exacerbations [3,4,16–18]. Previous *in vitro* studies with human primary cells suggest a plethora of roles of IL-1 family members in the inflammatory process [6]. However, the functional relevance of IL-1α and IL-1β for the pathology of COPD remains to be determined.

In this study, we used a recently described murine model in which pulmonary inflammation is triggered by CS and H1N1 to explore the relevance of IL-1α and IL-1β *in vivo* in a setting mimicking aspects of viral-induced COPD exacerbation [14]. We found that individual neutralization of IL-1α in CS/H1N1 challenged

mice had significant suppressive effects on inflammatory cell influx and KC levels in the lung. In contrast, individual neutralization of IL-1β did not provide significant reduction of inflammatory cells or cytokines/chemokines. Remarkably, the combined neutralization of IL-1α and IL-1β had additive (e.g., BAL cell influx, MCP-1) or over-additive (e.g., KC, IL-6) suppressive effects on CS/H1N1-induced airway inflammation and revealed a strongly pronounced anti-inflammatory activity. This data significantly extends a previous report investing the role of IL-1α in a related smoke/viral challenge model [3].

We found that treatment of H1N1-infected primary human bronchial epithelial cells obtained from a donor with COPD with IL-1α Abs resulted in significant protection from H1N1-induced loss in TER and in significant reduced levels of IL-6 and TNF-α whereas IL-1β Abs had no protective effect. These findings extend previous results reported in related *in vitro* settings where the IL-1R antagonist Anakinra was shown to suppress rhinovirus induced cytokine release from human primary epithelial cells [19] or where the same IL-1α neutralizing Ab that we used exerted anti-inflammatory activity in an airway epithelial/fibroblast coculture cell model [20].

In an additional *in vivo* study, application of an IL-1R1 Ab provided significant anti-inflammatory effects on BAL cell influx and lung mediator induction that were however inferior compared to inhibition of its soluble ligands, IL-1α and IL-1β. Combined neutralization of IL-1α/IL-1β had also significant protective effects on body weight loss which were superior when compared to the effects of IL-1R1 blockade. It may be that the observed suppressive effects of the IL-1 pathway inhibitors on body-weight loss are the consequence of reduced inflammation and/or may be based on direct effects on lipid/energy metabolism as described elsewhere [21,22].

Taken together, our work supports and extends previous findings that IL-1α might be a key player driving CS/virus induced airway inflammation [3,23] but also notes that concomitant blockade of IL-1β provides additional benefit. It also confirms and extends previous reports that blocking IL-1R1 is an efficient anti-inflammatory intervention in mouse CS and CS/virus models [3,4,11,12]. In addition, the results from our study clearly indicate that combined neutralization of IL-1α and IL-1β by Abs is superior to blockade of the IL-1R1, despite high lung target engagement and high dosing of the IL-1R1 Ab. It remains to be clarified whether differences in affinity, target distribution, or other yet to be defined parameters might account for the weaker activity of IL-1R1 Abs compared to treatment with combined IL-1α/IL-1β Abs or compared to IL-1R1−/− mice.

Notably, none of the Ab treatments resulted in increased viral loads in lung homogenate. In contrast, a reduced viral load was observed in the lungs of IL-1α/IL-1β Ab treated mice. One explanation might be that the epithelial barrier integrity is possibly less affected in less inflamed lung tissue and might hamper distribution of viral pathogens in the lung. In addition, combined neutralization of IL-1α/IL-1β by Abs also suppressed CS/H1N1-induced MUC5A/C and ICAM-1 mRNA expression. Both MUC5 A/C [24] and ICAM-1 [25,26] were previously linked to the pathogenesis of COPD. Excessive mucin and sputum production may negatively affect COPD progression [27]. ICAM-1 may contribute to the recruitment of neutrophils to the site of inflammation that are thought to play a central role in the inflammatory process of COPD and the development of emphysema [28,29].

Both an IL-1β Ab (Canakinumab) and an IL-1R1 (MEDI-8968) Ab were recently tested in Phase II studies in patients with COPD [5,13]. No clinically meaningful improvement in any lung function parameter was achieved by Canakinumab, although doses were used that provided efficacy in other inflammatory diseases. Therefore, it can be postulated that inhibition of IL-1β alone is not sufficient to provide clinical efficacy in COPD. The pre-clinical data generated in the present study supports this assumption. The apparently negative outcome of the anti-human IL-1R1 Ab MEDI-8968 is unexpected based on our pre-clinical data in this study and published data that suggest a crucial role of IL-1R1 and IL-1 signaling for CS- and CS/viral-induced lung inflammation [3,4,11,12]. We assume that our dosing regimen achieves close-to-maximal target engagement and efficacy in our model. However, we do not know how the clinical Abs compare to our tool Abs. One might also speculate that the translation of mouse models to the human COPD patient is not predictive. However, there are several lines of evidence (including our ALI studies with epithelial cells) that indicate that the IL-1 pathway is active in patients with COPD.

The data from our current study suggests that combined inhibition of IL-1α and IL-1β with individual Abs might be a preferable treatment approach. Moreover, targeting of additional potentially redundant IL-1 family members such as IL-18 might be required to achieve further efficacy, although neutralizing IL-18 did not provide convincing anti-inflammatory activity in our model (Supplemental Figs. S3–S4). In addition, there is emerging evidence that novel IL-1 family members such as IL-33 [31,32] and IL-36 [33] might also contribute to COPD pathology.

In conclusion, our results suggest that combined inhibition of both IL-1α and IL-1β is superior in reducing CS/H1N1-induced inflammation compared to individual neutralization of these cytokines or blockade of the IL-1R1 in mice. Our work supports the view that targeting the IL-1 pathway might also reduce CS/H1N1-induced airway inflammation in patients.

## Author contribution

*Participated in research design:* Bucher, Lamb, Wittenbrink, Fuchs, Jung, Erb, Peter.

*Conducted experiments:* Bucher, Keck, Mang, Przibilla.

*Contributed new reagents or analytic tools:* Wittenbrink, Fuchs, Schiele

*Performed data analysis:* Bucher, Keck, Mang, Przibilla, Peter.

*Wrote or contributed to the writing of the manuscript:* Bucher, Lamb, Jung, Erb, Peter.

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All authors are employees at Boehringer Ingelheim Pharma GmbH & Co. KG.

## Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.pupt.2017.03.008.

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**Publication #2: Importance of the IL-1 axis in *Haemophilus influenzae* stimulated $M_1$ macrophages driving transepithelial signaling**

For this publication I designed, conducted and analyzed most of the experiments and wrote the manuscript. I established the SAEC cultures, the macrophage cultures, the co-culture and the co-culture infection and did an in-depth characterization. The characterization was mainly done by determining the gene expression via RT-PCR, cytokine measurement, confocal microscopy and Next-generation sequencing (NGS). Techniques performed by me for the publication are confocal microscopy, continuous TEER measurements, measurements by MSD and gene analysis by RT-PCR.

I generated the hypothesis that IL-1 has a critical effect on epithelial barrier integrity. This was derived from observations made in the publication #1 and experiments that showed that the TEER drop in differentiated SAEC cultures was due to the presence of $M_{1.1}$ macrophages (macrophages stimulated with GM-CSF and NTHi). This coincided with high IL-1 levels in the cultures and was reversible by addition of IL-1 antibodies.

We found that pro-inflammatory macrophages can locally disturb the epithelial barrier, which leads to cytokines traversing the epithelium and potentially eliciting effects on the basolateral side.

This brings together the observed effects of IL-1 on the barrier integrity and cytokine secretion in the animal or DHBE cell model. Furthermore, it includes the observed interplay of macrophages and epithelial cells that have been demonstrated in the publication #3 about the effect macrophages can have on the injured epithelium.

# CORRESPONDENCE

## Importance of the IL-1 Axis in *Haemophilus influenzae*–stimulated $M_1$ Macrophages Driving Transepithelial Signaling

*To the Editor*:

Chronic obstructive pulmonary disease (COPD) is an inflammation-driven pulmonary disease in which lung function decline is accelerated by exacerbations linked to a number of pathogens, including nontypeable *Haemophilus influenzae* (NTHi) (1). Sensing of pathogens and inappropriate inflammation caused by airway macrophages may be a causal factor (2).

### Methods

All methods were performed in accordance with relevant guidelines and regulations.

Small airway epithelial cells (donor #408031, Lonza) were cultured and differentiated according to the Lonza Clonetics S-ALI air–liquid interface medium protocol. Peripheral blood mononuclear cells were isolated from healthy human blood by density gradient centrifugation in Ficoll-Paque PLUS-filled tubes (227288, Greiner) and cultured in XVIVO-10 medium supplemented with 100 ng/ml of granulocyte/macrophage-colony stimulating factor (GM-CSF) or M-CSF. The cellZscope automated cell monitoring system (nanoAnalytics GmbH) was used for continuous measurement of the transepithelial electrical resistance (TEER). The antibodies used were anti–IL-1α (MAB200; R&D), anti–IL-1β (canakinumab, Komptur Apotheke), IL-1 receptor antagonist (IL-1RA; 54592, Biomol), and anti–TNF-α (7321S, Cell Signaling). More details regarding the methods used are provided in the data supplement.

### Results

Macrophage classification traditionally describes GM-CSF (CSF2)–induced $M_1$-like ($M_{CSF2}$; proinflammatory) and M-CSF (CSF1)–induced $M_2$-like ($M_{CSF1}$; antiinflammatory) subtypes (3). Levels of GM-CSF are elevated in COPD (4), and monocyte-derived macrophages (MDMs) from patients with COPD produce more proinflammatory cytokines upon stimulation (5). Here, we show that GM-CSF–differentiated MDMs ($M_{CSF2}$) express the GM-CSF–dependent marker for airway macrophages, PPARγ (6), which is downregulated by NTHi stimulation (Figure 1A). This downregulation is consistent with previously observed levels in COPD and COPD exacerbations (7).

$M_{CSF2}$ macrophages dose dependently secreted more inflammatory cytokines (IL-1α, IL-1β, and TNF-α) than $M_{CSF1}$ macrophages in response to NTHi stimulation (Figures E1A–E1C in the data supplement). NTHi stimulation was also associated with a loss of phagocytic activity (Figures E1D and E1E), which may be associated with a loss of plasticity (8).

When stimulated with NTHi (Figure 1B) in a novel coculture system with differentiated human small airway epithelium at the air–liquid interface, apical $M_{CSF2}$ macrophages (but not $M_{CSF1}$ macrophages) disrupted the epithelial barrier, as evidenced by a reduction in TEER and an increase in FITC-dextran permeability (Figures E1F and E1G). This was associated with a dissociation of ZO-1 from the epithelial tight junctions, but only in close proximity to cocultured macrophages (Figure 1C). In the absence of macrophages, IL-1α, IL-1β (Figure 1G), and TNF-α, but not IL-4, IL-13, GM-CSF, M-CSF, or IL-10, were able to recapitulate the epithelial barrier disruption. Interestingly, the amount of IL-1 found in macrophage-conditioned medium is low, but it is plausible that local concentrations are much higher, particularly in the $M_{CSF2}$ macrophages (in which levels in the conditioned medium were higher than in $M_{CSF1}$ macrophage–conditioned medium), or even that cell–cell contact is crucial. It is also possible that the extracellular acidification observed in the $M_{CSF2}$ macrophage cultures (but not in the $M_{CSF1}$ macrophage cultures) drives elaboration of IL-1 (9) and/or directly increases epithelial permeability (10). This may explain the local epithelial tight-junction disruption only in the vicinity of macrophages. An analogous scenario has been reported in dermal tissue, where neutrophils preferentially extravasate in close proximity to perivascular macrophages (11).

In the presence of NTHi-stimulated $M_{CSF2}$ macrophages, blockade of IL-1α had no effect by itself, whereas blockade of IL-1β reversed the TEER decrease by 62% (Figure 1D; Table E1). In combination, anti–IL-1α and anti–IL-1β antibodies fully reversed the TEER decrease. These effects were only observed when the antibodies were applied to the apical side of the epithelium (and not the basolateral side) (Figure 1E). A similar effect of IL-1 on TEER has been studied with regard to the blood–brain barrier (12) and blood–testis barrier (13), and in a murine cigarette-smoke and viral-infection model in which IL-1α, rather than IL-1β, played a major role (14). The study by Bucher and colleagues (14) may suggest that viral inflammation is mediated by IL-1α and bacterial inflammation is mediated by IL-1β, as shown here. Canakinumab, an IL-1β–blocking antibody, did not reach its clinical efficacy endpoints in COPD (15). Our results and others (14) suggest that IL-1α and IL-1β have overadditive effects, and therefore blocking only IL-1β may not be adequate to achieve sufficient clinical efficacy. IL-1β release was blocked by the selective NALP3 inflammasome inhibitor MCC-50 (Table E2), suggesting that release was caspase dependent and did not occur not via necroptosis.

Herold and colleagues demonstrated that resident alveolar macrophages disrupted the epithelial barrier through IL-1β, leading to an influx of exudate macrophages in mice (16). However, contrary to our data, this effect was attributed to apoptotic epithelium (rather than tight-junction disruption) and was attenuated by exudate macrophage–derived IL-1RA. Interestingly, anakinra (IL-1RA) reversed the TEER decrease by only 42% (Figure 1D). This is consistent with the findings of Finch and colleagues, who achieved 60% macrophage influx inhibition in an LPS instillation mouse model (17).

Addition of an anti–TNF-α antibody was similarly effective on both the apical and basolateral sides (Figure 1F), suggesting that TNF-α transits the epithelium after IL-1–mediated barrier disruption, where it binds to basolateral receptors. A similar effect was previously observed in CACO-2 cells, where IL-8 was secreted differentially depending on the side of TNF-α stimulation (18).

NTHi stimulation of macrophages appears to be LPS dependent because the Toll-like receptor 4 inhibitor TAK-242 (1 μM) fully inhibited apical IL-1α, IL-1β, and TNF-α (86–96%) (Table 1). Inhibition of p38-mitogen-activated protein kinase

This letter has a data supplement, which is accessible from this issue's table of contents at www.atsjournals.org.

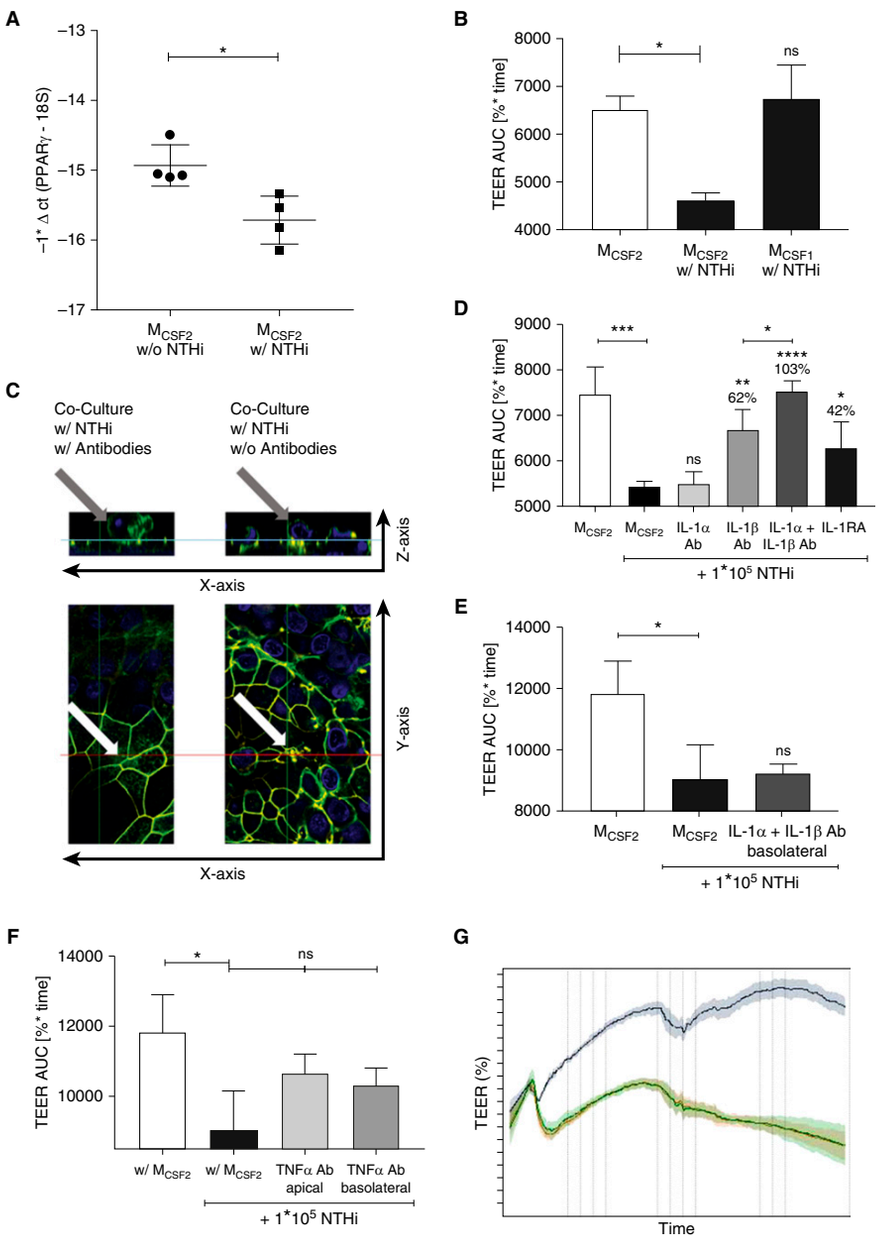


**Figure 1.** (*A*) Macrophages ($5 \times 10^4$) were stimulated with $1 \times 10^5$ nontypeable *Haemophilus influenzae* (NTHi) or no NTHi, and peroxisome proliferator-activated receptor γ (PPARγ) gene expression was determined by TaqMan (18S = 18S RNA). (*B*) Cocultures with $5 \times 10^4$ colony-stimulating factor 2 (CSF2) or CSF1 macrophages were stimulated with $1 \times 10^5$ NTHi from the apical side and the transepithelial electrical resistance (TEER) was measured. Values represent the mean area under the curve of the TEER over 48 hours. Mean + SD ($n = 3$). (*C*) Cocultures with NTHi and antibodies against IL-1α and IL-1β (left), and with NTHi and without antibodies against IL-1α and IL-1β (right) were stained and visualized via confocal microscopy, and a *Z*-stack of the culture was recorded (the upper panel displays the *Z*-stack; the light blue line represents the *x*/*y* plane in the upper panel, and the red line represents the view on the *z*/*x* plane in the lower panel). The cytoskeleton (F-actin) was stained in green, zona occludens 1 (ZO-1) was stained in yellow, and the nucleus (DAPI) was stained in blue. Cells "above" the tight junctions (ZO-1 signal) represent macrophages (actin and DAPI signal above the light blue line). The gray arrows point to apical macrophages, and the white arrows point to the tight junctions underneath the macrophages that were distorted when no antibodies were added to the coculture. (*D*) Cocultures were subjected to anti–IL-1α, anti–sIL-1β, a combination of both, or IL-1RA (100 nM each). Data are shown as mean + SD, $n \geq 3$. (*E*) Cocultures were stimulated with NTHi, IL-1 antibodies were added to the basolateral side of the culture, and TEER was measured for 48 hours. (*F*) Anti–TNF-α antibody (100 nM) was added to the cultures from the apical side or the basolateral side to demonstrate a difference in efficacy, and TEER was measured for 48 hours. Mean + SD, $n = 3$. (*G*) Small airway epithelial cells were incubated with 5 ng/ml of recombinant IL-1α or recombinant IL-1β. TEER was continuously measured for 48 hours and is represented as % TEER; the line shadows are the SD of $n = 3$. Asterisks represent statistical significance (*$P < 0.5$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$). Ab = antibody; AUC = area of the curve; ns = not significant; w = with; w/o = without.

**Table 1.** Inhibition of the TEER Reduction and Cyto-/Chemokines of NTHi-Stimulated Cocultures by p38-MAPK, TLR4 Inhibitors, or Prednisolone

| | TEER | | Apical IL-1α | | Apical IL-1β | | Apical TNF-α | |
|---|---|---|---|---|---|---|---|---|
| | % Inhibition | Mean AUC ± SD | % Inhibition | Mean conc. ± SD | % Inhibition | Mean conc. ± SD | % Inhibition | Mean conc. ± SD |
| Nonstimulated CTRL | — | 5170 ± 200 | — | 14.1 ± 3.43 | — | 3.6 ± 1.7 | — | 14.6 ± 1.3 |
| Stimulated CTRL | — | 3974 ± 243 | — | 157.7 ± 143.8 | — | 69.5 ± 28.7 | — | 312 ± 172.7 |
| p38-MAPK (SB203580) | 76**** | 4878 ± 239 | 58 | 75.4 ± 44 | 71** | 22.9 ± 9.1 | 61 | 129.8 ± 20.5 |
| TLR4 (TAK-242) | 62*** | 4710 ± 116 | 90 | 29 ± 8.6 | 86*** | 13 ± 8.6 | 96 | 24.6 ± 6.9 |
| Prednisolone | 0 | 4000 ± 118 | 10 | 144 ± 53.1 | 0 | 70.9 ± 25.5 | 0 | 491.4 ± 331.5 |

*Definition of abbreviations*: AUC, area under the curve; conc. = concentration; CTRL = control; MAPK, mitogen-activated protein kinase; NTHi = nontypeable *Haemophilus influenzae*; SD = standard deviation; TEER = transendothelial electronic resistance; TLR4 = Toll-like receptor 4.
Data are displayed as % inhibition of NTHi-stimulated and nonstimulated cocultures. Mean AUC ± SD is displayed as [% × time]; $n = 4$. Cytokine values are displayed as pg/ml ± SD; $n = 4$. Asterisks represent statistical significance (**$P \leq 0.01$; ***$P \leq 0.001$; ****$P \leq 0.0001$).

(p38-MAPK) has been suggested as a therapeutic strategy to treat COPD. The p38 inhibitor SB203580 (1 μM) resulted in a somewhat lower inhibition of apical IL-1α, IL-1β, and TNF-α (58–71%) (Table 1). Clinical evaluations of p38-MAPK inhibitors in an LPS challenge model showed reduced neutrophil counts in sputum (19) and improved lung function in patients with COPD (20). However, GlaxoSmithKline very recently terminated the development of their p38 inhibitor, losmapimod, for lack of efficacy in patients with COPD. Blocking this pathway resulted in an approximately two-thirds inhibition of macrophage-derived mediators in our system, suggesting that greater inhibition may be required to achieve clinical efficacy (21). Interestingly, prednisolone (1 μM) had no effect on any parameter (Table 1), suggesting a steroid insensitivity in this model system, analogous to the refractory treatment responses to steroids reported by Barnes (2).

In conclusion, for the first time, we were able to show the importance of the IL-1 axis in bacterial/macrophage induction of epithelial permeability in a fully differentiated primary human macrophage–epithelial cell coculture system. The COPD-relevant $M_{CSF2}$ (but not $M_{CSF1}$) MDMs stimulated with NTHi locally perturbed the epithelial barrier to enable transepithelial chemokine/cytokine transit. This effect was mediated by NALP3; macrophage-derived IL-1α, IL-1β, and TNF-α; and p38-MAPK. ■

**Author disclosures** are available with the text of this letter at www.atsjournals.org.

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## ONLINE SUPPLEMENT

## Methods

## Cell Culture

Small Airway Epithelial Cells (Donor #408031; 57 year old female; Caucasian; passage 2; $1.1*10^6$ cells/vial; seeding efficiency: 85%; doubling time: 37 h; viability: 79%) were purchased from LONZA (Verviers, Belgium), cultured and differentiated following the LONZA Clonetics™ S-ALI™ air-liquid interface medium protocol. As stated by Lonza, the cells were isolated from donated human tissue after obtaining permission for their use in research applications by informed consent or legal authorization.

Cells were thawed and seeded into a T175 NUNC flask (178883, Thermo Fischer) on day -8, medium was changed on day -7 and on day -4, the cells were trypsinised off the T-Flask and seeded on Corning Transwell filters (CLS3470, Corning) at 22.000 cells/well. S-ALI Differentiation Basal Medium (CC-3282 & CC-4124, Lonza) was changed apically and basolaterally on day -3. On day 0 the apical medium was removed and the basolateral growth medium was substituted with S-ALI Differentiation Medium (CC-3282 & CC-4124, Lonza). The cells were washed on the apical side with PBS to remove growth factors. The cells were then differentiated for at least 4 weeks with basolateral medium changes 3 times a week. Cells were washed apically every week to remove excess mucus. .

PBMCs were isolated from healthy human blood by density gradient centrifugation @ 800 x g for 20 minutes in Ficoll-Paque™ PLUS filled Greiner Separation Tubes (227288; Greiner).

$CD14^+$ monocytes were isolated from PBMCs using the Miltenyi Monocyte Isolation Kit II, human (Miltenyi Biotec; Bergisch Gladbach, Germany) achieving purity of about 90%. Between $5*10^6$ and $8*10^6$ $CD14^+$ Monocytes were seeded into 10 cm Thermo Scientific™ Nunc™ dishes with UpCell™ Surface (Thermo Fisher) in 10 mL XVIVO-10 medium supplemented with 100 ng/mL GM-CSF ($M_1$) or M-CSF ($M_2$).

After 6 days 5 mL XVIVO-10 medium supplemented with 100 ng/mL GM-CSF or M-CSF was added. Subsequently, the medium was changed every 2-3 days by adding 6 mL of the used medium back into the dish with 6 mL fresh medium. Cells were detached by cooling the plates to room temperature.

Cells were washed twice with $Ca^{++}/Mg^{++}$ free PBS and cell counts were determined in a Countess™ (Invitrogen) cell counter.

(co-culture setup visualised in Supplemental Figure 1)

## ELISA/MSD

For cytokine measurement the Mesoscale Discovery V- and U-PLEX multiplexing technology was used. V-PLEX plates were used in the Chemokine and Pro-Inflammatory configuration. U-PLEX plates were individually spotted with the antibody pairs against the desired analytes. Assays were performed according to the manufacturer's instructions.

## Microscopy

Cells on transwells were fixed with 1:1 Acetone/Methanol and incubated with ZO-1 primary antibody (1:200 rabbit-anti-ZO-1; invitrogen 402200); secondary antibody against rabbit IgG coupled to AF568 (1:400 anti-rabbit-IgG-AF568; Thermo Fisher). ActinGreen™ 488 ReadyProbes® (R37110; Thermo Fisher) were used to stain the cytoskeleton. ProLong® Gold Antifade Mountant with DAPI (P36935; Thermo Fisher) was used to counterstain the nuclei. Pictures were taken with a Zeiss LSM710 confocal microscope and ZEN software.

## TEER Measurement

The CellZScope Automated Cell Monitoring System (nanoAnalytics GmbH; Münster, Germany) was used for continuous measurement of TEER. The 24 wells in the machine were filled with 800 µL of S-ALI Differentiation Medium and warmed up in the Incubator. Transwell filters with cells were added to the wells and 200 - 250 µL XVIVO-10 Medium was added to the apical side.

For the co-culture experiments 175 µL XVIVO-10 medium was added initially and then 25 µL XVIVO-10 medium containing 5x concentrated antibodies. A further 25 µL were added that contained $5*10^4$ macrophages. After 45 minutes or more, $1*10^5$ heat killed NTHi were added in 25 µL XVIVO-10 medium.

The incubation period was 48h.

### Compounds & Antibodies

Antibodies and inhibitors used in the experiments were, anti-IL1α (MAB200; R&D); anti-IL1β (Canakinumab; Komptur Apotheke); IL1RA (rHuIL1RA; Biomol 54592); 6α-Methylprednisolone 21-hemisuccinate sodium salt (“steroid”, Sigma); p38-MAPK (SP203580; Sigma); TLR4 (TAK-242, resatorvid); NLRP3 (MCC-950)

## Calculations & Statistics

TEER values are displayed as AUC * % (Area under the curve of continuously measured, normalized TEER values multiplied with the time). This means that the curves were normalized at the time NTHi was added to the culture (t=0h) and then the AUC from t=0h to t=48h was calculated.

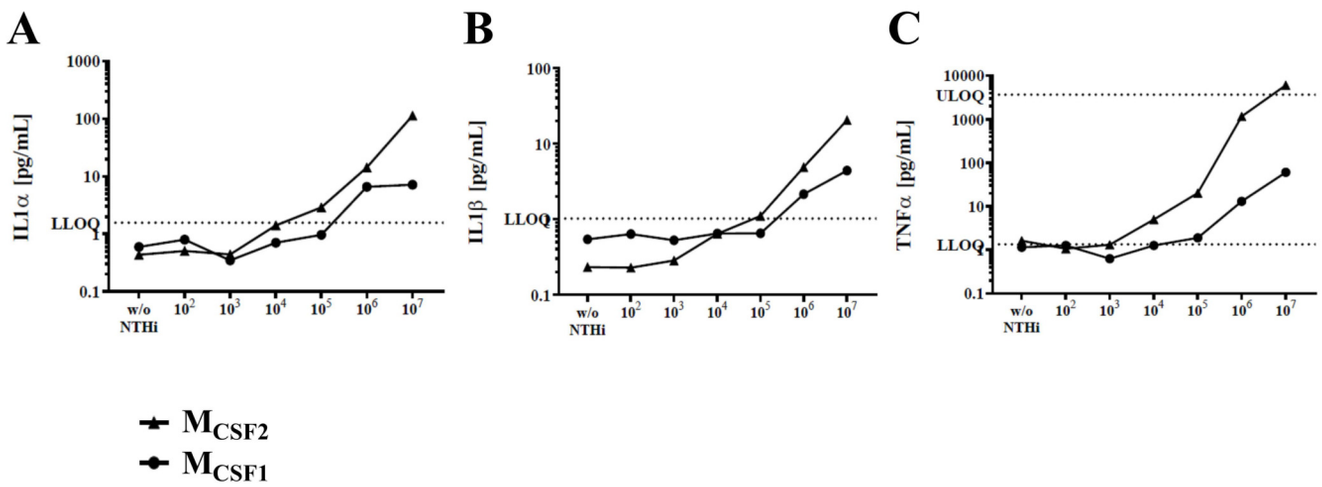
For statistical analyses Graph Pad Prism Software for Windows version 7 was used. Significance levels are shown as * $p \leq 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$ or “ns” for not significant ($p > 0.05$); ANOVAs were corrected for multiple comparisons with a Tuckey or Sidaks correction.

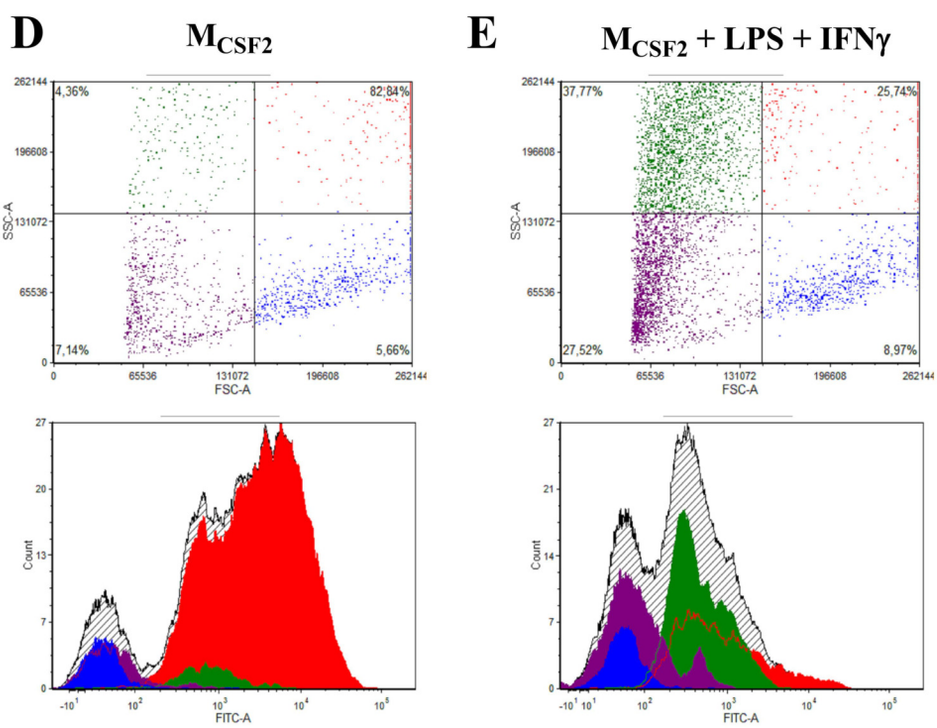
**Supplementary Table 1:** total TEER values measured in ohm after 48 h including the SD of n=4. $M_1$ = Co-culture w/o NTHi; $M_{1.1}$ = Co-culture w/ NTHi

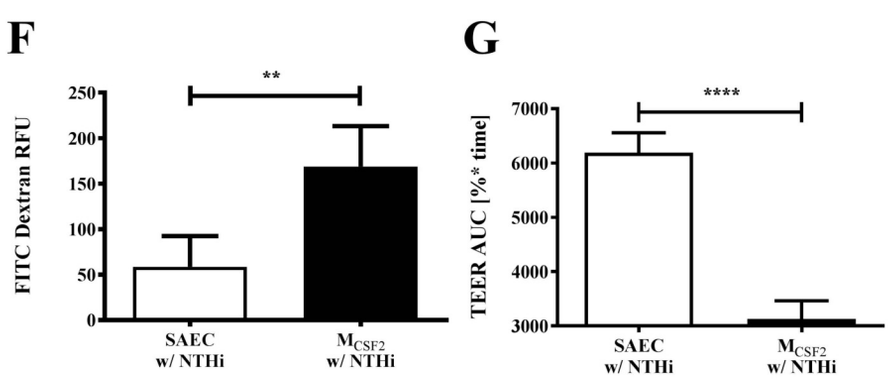
| $M_1$ | | $M_{1.1}$ | | $M_{1.1}$ and Anti-IL-1α | | $M_{1.1}$ and Anti-IL-1β | | $M_{1.1}$ and Anti-IL-1α/ β | | $M_{1.1}$ and IL-1RN | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| TEER [ohm] | SD | TEER [ohm] | SD | TEER [ohm] | SD | TEER [ohm] | SD | TEER [ohm] | SD | TEER [ohm] | SD |
| 998 | 140 | 529 | 19 | 717 | 302 | 768 | 194 | 1050 | 167 | 716 | 158 |

**Supplementary Table 2:** Inhibition of TEER and cyto-/chemokines by NALP3 inhibition. Data are displayed as % inhibition of NTHi stimulated and non-stimulated co-cultures. Mean AUC ± SD is displayed as [%*time]; n=4. Cytokine values are displayed as pg/mL ± SD; n=4. Asterisks represent statistical significance; * p≤0.5; ** p≤0.01

| | TEER | | Apical IL-1α | | Apical IL-1β | | Apical TNFα | |
|---|---|---|---|---|---|---|---|---|
| | % inhibition | mean AUC ± SD | % inhibition | mean conc ± SD | % inhibition | mean conc ± SD | % inhibition | mean conc ± SD |
| Non-stimulated CTRL | - | 5170 ± 200 | - | 14.1 ± 3.43 | - | 3.6 ± 1.7 | - | 14.6 ± 1.3 |
| Stimulated CTRL | - | 3974 ± 243 | - | 157.7 ± 143.8 | - | 69.5 ± 28.7 | - | 312 ± 172.7 |
| NALP3 (MCC-950) | 38* | 4426 ± 199 | 0 | 175.1 ± 116.5 | 68** | 25 ± 8.4 | 15 | 266.3 ± 56 |


A
B
C
IL1α [pg/mL]
IL1β [pg/mL]
TNFα [pg/mL]
LLOQ
ULOQ
w/o NTHi
M_CSF2
M_CSF1


D
M_CSF2
E
M_CSF2 + LPS + IFNγ
4,36%
82,84%
7,14%
5,66%
37,77%
25,74%
27,52%
8,97%
SSC-A
FSC-A
Count
FITC-A


F
G
FITC Dextran RFU
TEER AUC [%* time]
SAEC w/ NTHi
M_CSF2 w/ NTHi

**Publication #3: Opposing effects of *in vitro* differentiated macrophages sub-type on epithelial wound healing**

For this publication I supervised, designed, conducted and analyzed the experiments that were part of a maters thesis. For the experiments the SAEC cultures, the macrophage cultures and the co-cultures were used that had been established by me. The main part was to guide the master's student that conducted the masters' thesis in our department. As a topic for the masters' thesis, we followed up on various experiments that had been done by me during the start of my thesis.

For this publication we hypothesized that macrophages are involved in the regeneration of wounds that are physically introduced to differentiated epithelial cell layers. For that we introduced scratch wounds to the epithelial layer and added different macrophage subtypes to the apical side of the injured epithelium.

The experiments provided novel insights into the way that epithelia regenerate and how macrophages can have effects on the regenerating epithelium.

PLOS ONE

RESEARCH ARTICLE

# Opposing effects of *in vitro* differentiated macrophages sub-type on epithelial wound healing

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## Abstract

Inappropriate repair responses to pulmonary epithelial injury have been linked to perturbation of epithelial barrier function and airway remodelling in a number of respiratory diseases, including chronic obstructive pulmonary disease and idiopathic pulmonary fibrosis. We developed an *in vitro* mechanical scratch injury model in air-liquid interface differentiated primary human small airway epithelial cells that recapitulates many of the characteristics observed during epithelial wound injury in both human tissue and small animal models. Wound closure was initially associated with de-differentiation of the differentiated apical cells and rapid migration into the wound site, followed by proliferation of apical cells behind the wound edge, together with increases in FAK expression, fibronectin and reduction in PAI-1 which collectively facilitate cell motility and extracellular matrix deposition. Macrophages are intimately involved in wound repair so we sought to investigate the role of macrophage sub-types on this process in a novel primary human co-culture model. $M_1$ macrophages promoted FAK expression and both $M_1$ and $M_2$ macrophages promoted epithelial de-differentiation. Interestingly, $M_{2a}$ macrophages inhibited both proliferation and fibronectin expression, possibly via the retinoic acid pathway, whereas $M_{2b}$ and $M_{2c}$ macrophages prevented fibronectin deposition, possibly via MMP expression. Collectively these data highlight the complex nature of epithelial wound closure, the differential impact of macrophage sub-types on this process, and the heterogenic and non-delineated function of these macrophages.



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## Introduction

Inappropriate repair responses to macro- and micro-pulmonary epithelial injury have been linked to perturbation of epithelial barrier function and airway remodelling, possibly as a

design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Competing interests:** IC, JS and DJL are employees of Boehringer Ingelheim Pharma GmbH & Co. FG is an employee of C. H. Boehringer Sohn AG & Co. KG. SM, JAG and NP receive grant support from Boehringer Ingelheim Pharma GmbH & Co. This does not alter our adherence to PLOS ONE policies on sharing data and materials.

consequence of epithelial-mesenchymal transition, in a number of respiratory diseases, including chronic obstructive pulmonary disease (COPD) [1] and idiopathic pulmonary fibrosis (IPF) [2]. Whilst the precise mechanisms that are evoked during epithelial wound healing remain controversial [3], it is believed that cells at the wound edge dedifferentiate and flatten enabling migration over the damaged area. The epithelial basal cells then proliferate and the wound becomes filled with undifferentiated cells [4]. Subsequently, it is believed that a process of de-differentiation takes place and extracellular matrix proteins such as fibronectin are produced, which form the scaffold for the regenerated epithelium [5–7] although it may take several days before the injury is fully repaired [4].

Tissue macrophages are often described as existing in polarised states often termed $M_1$ and $M_2$, although recent data suggest that such cells exist in a more heterogeneous state [8]. In IPF, a number of groups have described lung macrophages as possessing $M_2$-associated phenotypes, such as CD163 and it has been discussed that such macrophages are associated with a tissue remodelling phenotype. In contrast, macrophages have been associated with COPD exacerbations [9] and that the inflammatory response may promote an $M_1$ phenotype. Indeed, granulocyte-macrophage colony stimulating factor (GM-CSF), an $M_1$ macrophage differentiation factor, and product of cells activated during inflammation, are elevated during COPD [10, 11].

In this study, we sought to model and characterise the pulmonary epithelial response to injury using mechanical scratching of primary human small airway epithelial cells in air-liquid interface culture as a test system. Furthermore we aimed to explore the role of primary human macrophages differentiated *ex vivo* into different phenotypes on this process in a novel primary human cell co-culture system.

## Results

### Characterisation of epithelial injury

The air-liquid interface differentiated human small airway epithelial cell culture inserts were injured by scratching the surface with 10 μL, 20 μL or 200 μL plastic pipette tip and monitoring the transepithelial electrical resistance (TEER; Fig 1A). TEER completely recovered within 40 hours of scratching with a 10 μL pipette tip, but recovered to only 70–80% control after 44 hours of scratching with a 20 μL or 200 μL pipette tip. A 200 μL pipette tip was chosen for subsequent experiments, because it produced a uniform scratch comprising approximately 20% of the total cell area. The mean width of the scratch was measured using the CellIQ (Fig 1B). In 48 consecutive scratches, the mean width was 68.4 ± 5.4 pts (Fig 1C). Next, we measured the dynamics of closure of the scratch by measuring the scratch size at different time points. The width of the scratch was reduced at 6 hours, 24 hours and 48 hours by 10%, 66% and 97% respectively (Fig 1D). The response to injury appeared to be initiated from a series of "hot spots" in cell clusters adjacent to the wound edge, rather than a homogenous response along the wound edge (S1 Video). We observed that, if the underlying collagen coating was damaged during the scratch (lower scratch border, S2 Video) then the response to injury was impaired along the site of damage until wound closure was almost complete.

Immunocytochemistry staining reveals KRT5-positive basal cells are prominent at the leading edge of wound repair 6 hours after injury, but thereafter are not seen at the leading edge (Fig 2A). Wound closure between 24 and 72 hours appears to be associated with a reduction in expression of both tight junction associated molecules (CLDN3, OCLN) and differentiation markers for ciliated cells (FOXJ1), club cells (SCGB1A1), columnar epithelial cells (TFF3), and α-smooth muscle actin (ACTA2) (Table 1).

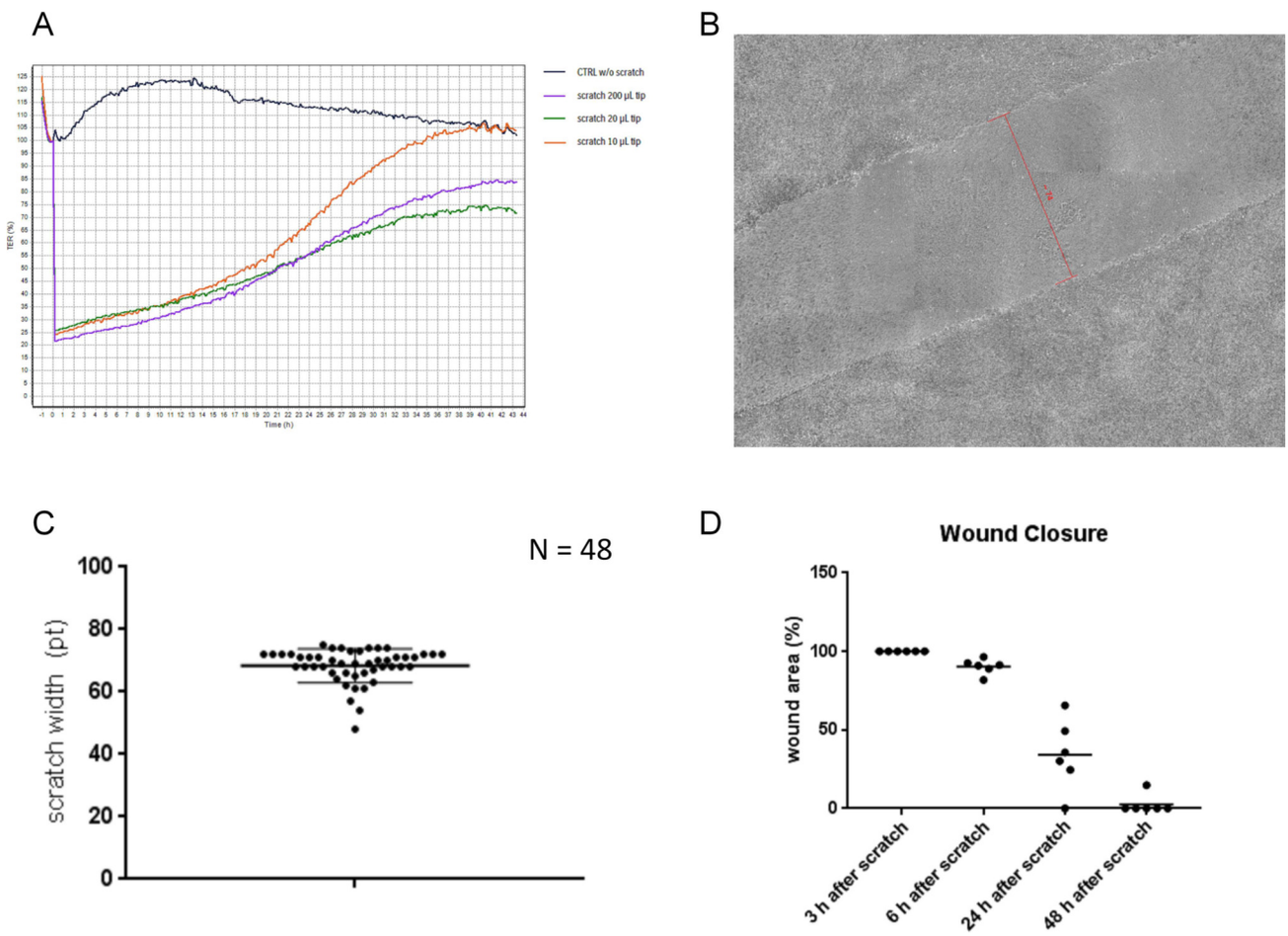


**Fig 1. TEER development and Scratch width measurement. A** TEER development measured in the cellZScope over time using no (black line), a 200 µL (orange line), a 20 µL (green line) and a 10 µL (violet line) pipet tip. **B** Microscopic image (CellIQ) of a scratch introduced with a 200 µL pipette tip (width (74 pts) of the scratch indicated by the red line) **C** scratch width (mean width 68.38 ± 5.39 pts) measured and plotted to demonstrate reproducibility of the scratch (n = 48) **D** time course of the closure of the scratch (n = 6)

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Interestingly, Ki67-positive proliferating cells are observed behind the leading edge from 6 hours onwards (Fig 2A) and the increase in cellular proliferation was confirmed by a significant increase in Ki67 mRNA observed 48 and 72 hours after injury (Table 1). Both focal adhesion kinase (FAK) and fibronectin staining was present on the leading edge of the 24 and 48 hours after injury and fibronectin (FN1) was still present 72 hours after injury when the wound had been physically closed (Fig 2B). This was accompanied by an increase in soluble fibronectin 48 and 72 hours after injury (Fig 2C).

## Characterisation of macrophage phenotype for co-culture experiments

Monocyte-derived macrophages were differentiated into "$M_1$" and "$M_2$" phenotypes by culturing with GM-CSF and M-CSF respectively for 7 days. "$M_2$" macrophages were incubated with IL-4/IL-13, immune complexes/LPS, or IL-10 to generate "$M_{2a}$", "$M_{2b}$" and "$M_{2c}$" sub-phenotypes. CD68 was uniformly expressed across the different sub-types, whereas CD80 was expressed on at low levels on all macrophages sub-types. CD163 was predominantly expressed on "$M_{2c}$" macrophages.

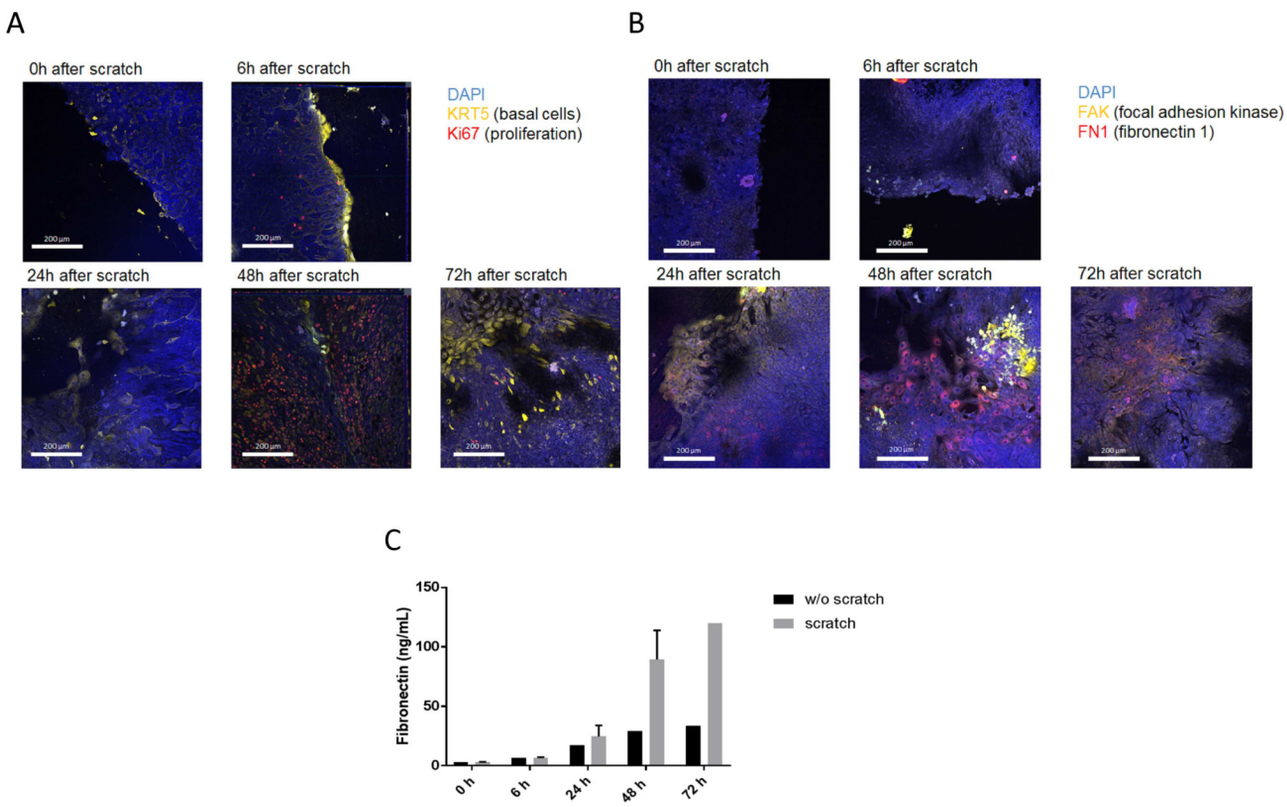


**Fig 2. IHC of scratched cells and fibronectin expression. A** IHC microscopic images of scratched cells at t = 0h, 6h, 24h, 48h and 72h; DAPI in blue, KRT5 in yellow, Ki67 in red. **B** IHC microscopic images of scratched cells at t = 0h, 6h, 24h, 48h and 72h; DAPI in blue, FAK in yellow, FN1 in red. **C** ELISA of FN1 in the supernatant after 0h, 6h, 24h, 48h and 72h with and without scratch.

https://doi.org/10.1371/journal.pone.0184386.g002

## Effect of macrophage phenotype on epithelial injury response

Addition of non-stimulated "$M_1$" macrophages increased F-actin staining in epithelial cells at both 24 and 72 hours post-injury (Fig 3A, panels 3 and 4). "$M_2$" macrophages also increased F-actin staining, but only 72 hours post-injury (Fig 3A, panel 6). "$M_2$" macrophages also suppressed FAK staining both 24 and 72 hours post-injury (Fig 3A, panels 5 and 6).

**Table 1. Regulation of selected genes in small airway epithelial cells after injury.**

| Molecule association | Gene | Fold regulation in expression after injury | | | |
|---|---|---|---|---|---|
| | | 6h | 24h | 48h | 72h |
| Tight junctions | CLDN3 | 1,29 | -1,98 | -2,54 | -1,25 |
| | OCLN | 2,08 | -1,50 | -1,55 | -1,19 |
| Differentiation | FOXJ1 | 1,69 | -1,77 | -1,38 | -1,60 |
| | SCGB1A1 | 1,79 | -3,66 | -2,69 | -2,79 |
| | TFF3 | 1,16 | -1,78 | -2,36 | -1,97 |
| | ACTA2 | 1,60 | -1,09 | -1,27 | -1,30 |
| Proliferation | Ki67 | 1,24 | 1,44 | 6,26 | 10,81 |
| Extracellular matrix | FN1 | -1,74 | -1,80 | 1,25 | 2,72 |

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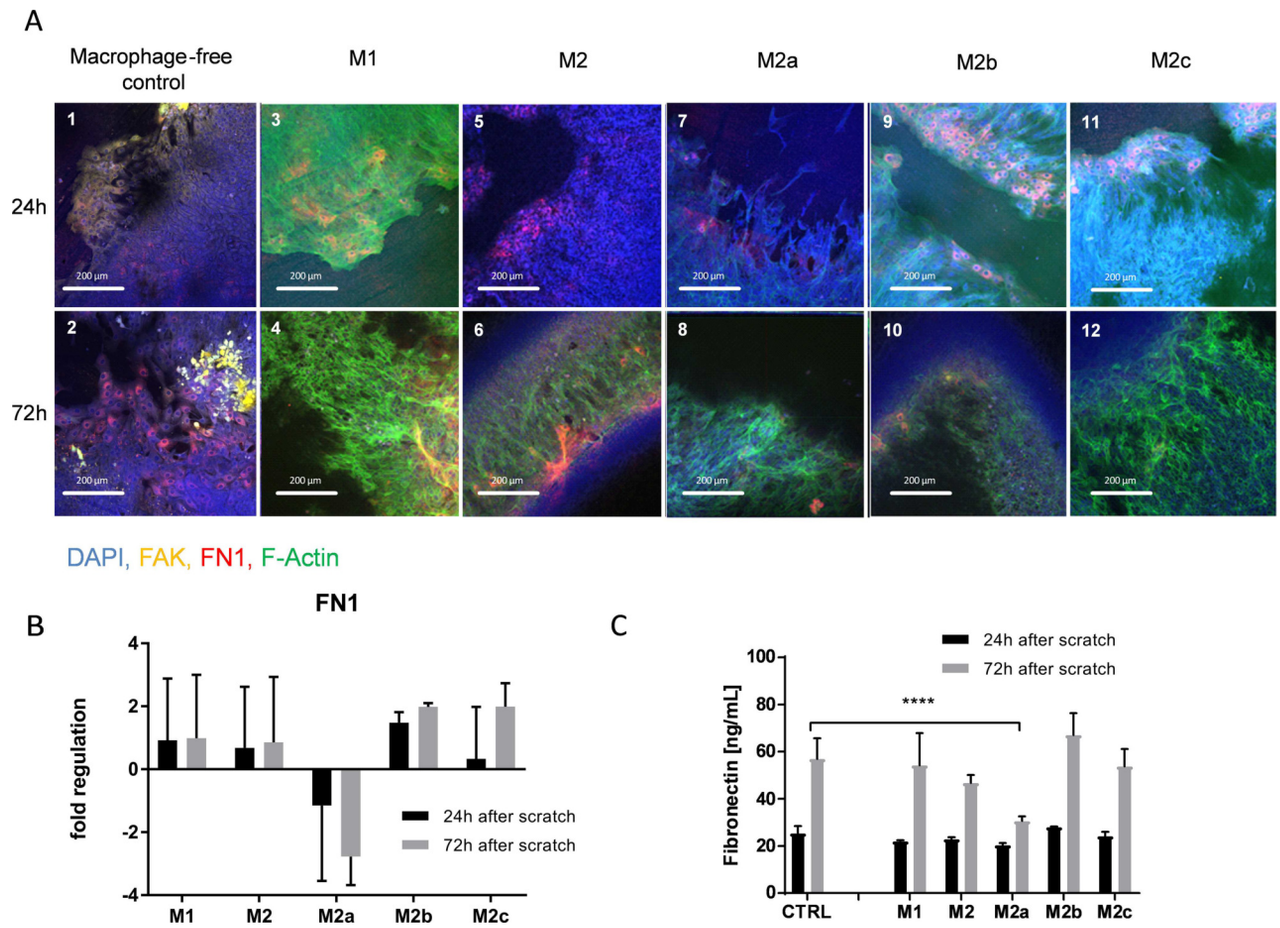


**Fig 3. IHC of scratched cells and FN1 regulation/expression. A** scratched epithelial cells incubated with different macrophage subtypes after 24h and 72h; DAPI stained in blue, FAK stained in yellow, FN1 stained in red, F-Actin stained in green. **B** FN1 mRNA regulation in the co-culture cell lysates measured after 24h and 72h Data are displayed as fold regulation compared to control medium containing macrophage maturation factors; Data is expressed as mean ± SD; n = 3 **C** FN1 expression measured in the supernatants via ELISA after 24h and 72h; Data is expressed as mean ± SD; n = 3 (**** = p>0.0001)

https://doi.org/10.1371/journal.pone.0184386.g003

Fibronectin staining in the epithelium after injury was also reduced by "$M_{2a}$" macrophages 24 and 72 hours after injury (Fig 3A, panels 7 and 8). Interestingly, intracellular fibronectin was observed 24 hours after injury in epithelial cells co-cultured with "$M_{2b}$" and "$M_{2c}$" macrophages, but was not apparent 72 hours after injury (Fig 3A, panels 9–12). Epithelial fibronectin gene expression was reduced by the "$M_{2a}$" macrophage subtype, but not with "$M_{2b}$" or "$M_{2c}$" subtypes (Fig 3B) and soluble fibronectin was also decreased in the co-cultures with "$M_{2a}$" but not "$M_{2b}$" or "$M_{2c}$" cultures 72 hours after injury (Fig 3C).

Following injury, epithelial cells co-cultured with "$M_{2a}$", "$M_{2b}$" and "$M_{2c}$" macrophages appeared to be less de-differentiated as evidenced by the lack of loss of MUC5AC, CLDN3, OCLN and TFF3 (Fig 4). There was also a reduced loss of SERPINE1 suggesting that the urokinase-mediated matrix degradation system was not impaired. Interestingly, there was a significant reduction in epithelial cell proliferation, as measured by Ki67 gene expression, following injury and co-culture with "$M_{2a}$" macrophages, but not with "$M_{2b}$" or "$M_{2c}$" (Fig 4).

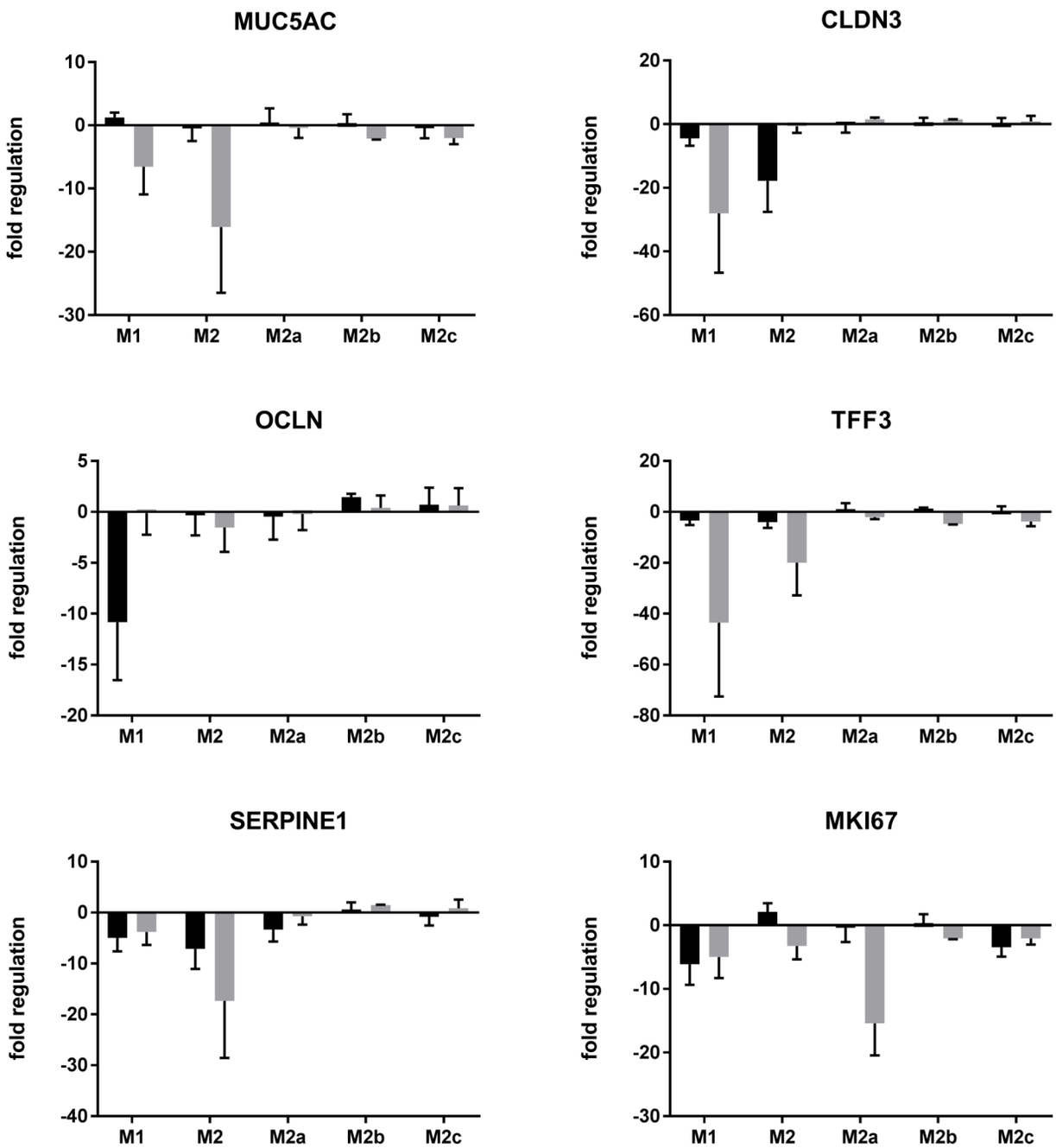


**Fig 4. mRNA regulation in the co-cultures.** mRNA expression levels in lysates of the co-culture after 24h and 72h for MUC5AC, CLDN3, OCLDN, TFF3, SERPINE1 and MKI67/Ki67; Data are displayed as fold regulation compared to control medium containing macrophage maturation factors; Data is expressed as mean ± SD; n = 3

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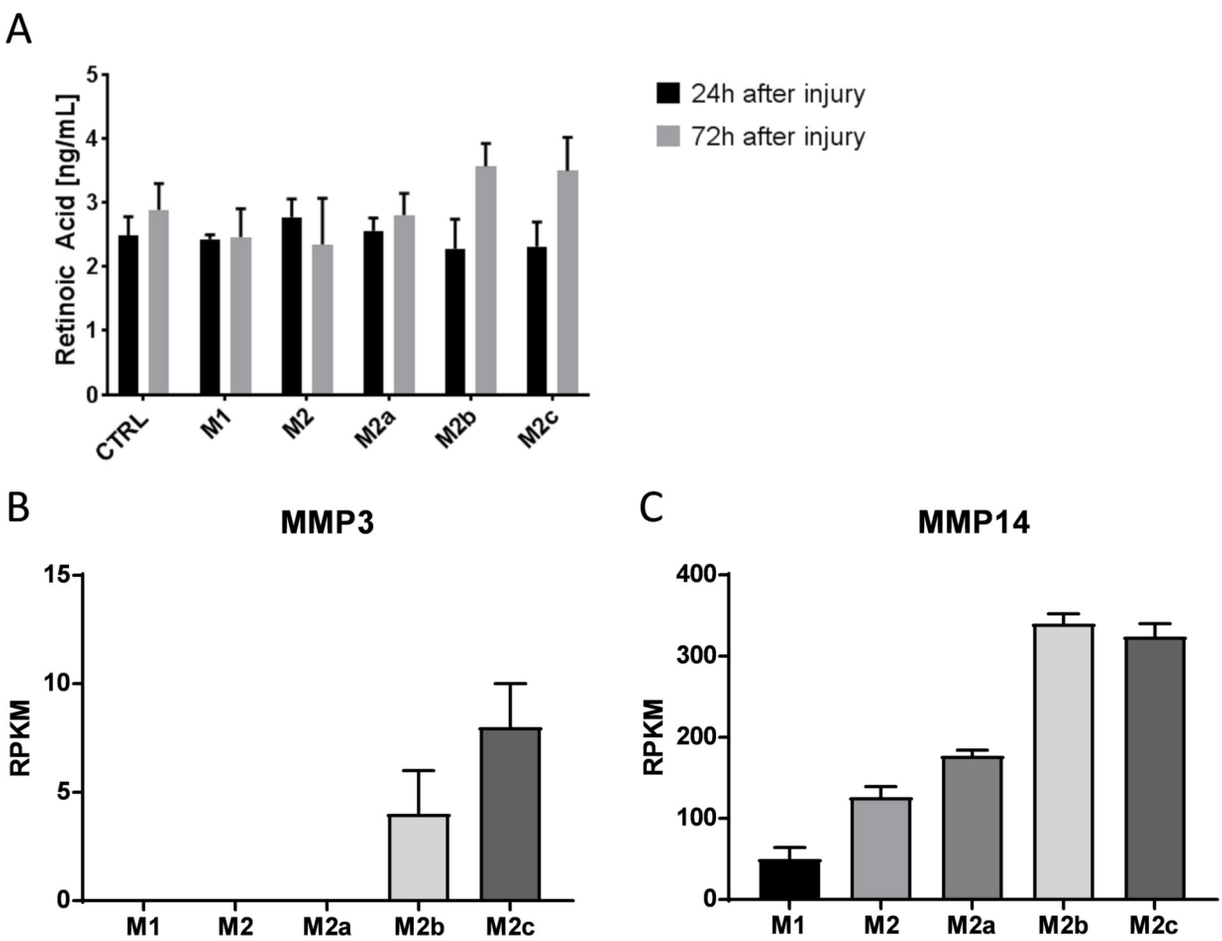


**Fig 5. Retinoid acid amount and MMP3/MMP14 expression. A** Retinoic acid concentration in supernatants of different macrophage subtypes was measured by ELISA **B** MMP3 and **C** MMP14 expression in different macrophage subtypes was measured by TaqMan RPKM = Reads per kilobase per million mapped reads; Data is expressed as mean ± SD; n = 3

https://doi.org/10.1371/journal.pone.0184386.g005

In order to understand the effects of the macrophage sub-types on epithelial response to injury, we looked for factors known to modulate fibronectin expression and/or cross-linking/degradation. The "$M_{2b}$" and "$M_{2c}$" macrophages secreted more retinoic acid, compared with "$M_1$", "$M_2$" and "$M_{2a}$" into the medium (Fig 5A). Furthermore, "$M_{2b}$" and "$M_{2c}$" macrophages were the only subtypes that expressed MMP3 (Fig 5B) and expressed significantly higher levels of MMP14, compared with "$M_1$", "$M_2$" and "$M_{2a}$" sub-types (Fig 5C).

## Discussion

In this study we used primary human small airway epithelial cells differentiated in air-liquid interface culture as a model of the pulmonary epithelium. These cells form a bilayer consisting of basal cells below and differentiated apical cell layer that consists of ciliated columnar epithelial cells and secretory cells. Manual scratching of differentiated small airway epithelial cells results in a reproducible cell-free area that features disrupted cells at the wound edges, which is repaired in a consistent manner by neighbouring cells. Real-time monitoring of epithelial wound repair, revealed a highly migratory process. Cells in the vicinity of the scratch were

observed to migrate into the space, a motion that seems to start from several "hot spot" origins, which is consistent with the observations of Erjefält *et al.* [4]. Even after the gap was apparently physically closed, approximately 30 hours after injury, cell movement was still apparent. This was consistent with the recovery of TEER, which was not complete until approximately 48 hours after injury, presumably a measure of the recovery of epithelial integrity and the generation of new tight junctions, despite the fact that the expression of both OCLN and CLDN3 were still down-regulated 72 hours after injury (although mRNA levels as a labile precursor do not necessarily reflect protein levels). Interestingly, the basal cells did not stain positively for the proliferation marker Ki67, but the proliferating cells appeared to reside in the apical layer behind the leading edge of the wound, and only at later time points (the signal was highest 48 hours after injury). This was confirmed by Ki67 mRNA levels that were elevated 48 and even more so at 72 hours post-injury. Furthermore, basal cells were initially observed on the leading edge of the wound at early time points (up to 6 hours), but not thereafter. This would indicate that the initial physical wound closure and recovery of epithelial barrier integrity is predominantly facilitated by migration of differentiated cells from the apical layer into the damaged area, and once barrier integrity is restored, distal apical cells behind the wound proliferate to replenish cell numbers. This observation is contrary to reports that suggest that basal cells play a more dominant role in barrier recovery following oxidative [12, 13] or chemical [14, 15] damage. It could be speculated that this apparent discrepancy is in response to different types of injury and indeed small animal models of mechanical epithelial injury appear to indicate that an initial key event is the cells bordering the lesion to dedifferentiate and flatten which then appear to migrate inwards and over the denuded area to restore the barrier function of the epithelium [16–22]. Interestingly, an intact collagen-coated membrane appears essential for the initial migratory process, although cells appear to be able to eventually cross a damaged membrane as cells on the opposing wound edge become closer, suggesting perhaps a soluble mediator may be responsible. It has been reported that cell motility can be regulated at least in part by Focal Adhesion Kinase [23]. In the immunocytochemistry, FAK was up-regulated on the leading edge of the wound 24 hours after injury which is consistent with the motility observed in the time-lapse videos. We also observed de-differentiation in the apical cells following injury, as described by the down-regulation of FOXJ1 (ciliated cell marker), SCGB1A1 (club cell marker), TFF3 (columnar epithelial cell marker) and ACTA2 (smooth muscle actin), which is also consistent with the observations reported in the small animal models. We also observed a concomitant increase in both mRNA and protein levels of fibronectin following injury. Initially this was apparently intracellular on the leading edge of the wound, but later also apparently deposited extracellularly. Generally, accumulation of extracellular matrix proteins such as collagen or fibronectin is a physiological response to injury [24]. Both soluble and insoluble fibronectin have been shown to increase alveolar epithelial wound healing in an *in vitro* model, increasing cell motility and spreading, but not apparently cellular proliferation [25].

Next we wanted to assess the effect of differentiated macrophage sub-types on the epithelial wound-repair process. Despite numerous attempts, it was not technically possible to determine whether different macrophage per se, or different sub-types affected the rate of wound closure. Nevertheless, there was clear impact of macrophages on some of the epithelial phenotypic characteristics during wound healing. $M_1$, but not $M_2$, macrophages enhanced the expression of FAK. FAK is reported to be not only associated with enhanced motility, but also epithelial-mesenchymal transition [23], an event linked with COPD [1] and IPF [2]. This is somewhat contradictory to the dogma of $M_2$ like macrophages possessing a more pro-fibrotic phenotype [26]. It has been reported that TNF-α induces cytoskeletal rearrangement and FAK activation in endothelial cells via a RhoA-dependent process [27] and that Rho GTPase

mediates this permeability [28]. We found that $M_1$ macrophages secreted more TNF-α, compared with $M_2$, $M_{2a}$, $M_{2b}$ and $M_{2c}$ macrophages (S1 Fig) and that TNFα also mediated increases in epithelial permeability (S2 Fig), suggesting that the increases in FAK observed may be a consequence of activating the TNF-α pathway. Interestingly, $M_1$ macrophages were also associated with a greater down-regulation of epithelial Muc5AC, which has also been demonstrated to be down-regulated during EMT [29], but down-regulation was even more pronounced in the co-culture with $M_2$ macrophages. Both $M_1$, and to a lesser extent $M_2$ macrophages, were associated with loss of tight junction protein expression (CLDN3 and OCLN) and the columnar epithelial cell marker TFF3 suggesting promotion of epithelial de-differentiation. SERPINE1 expression was up-regulated following injury, but down-regulated in the presence of both $M_1$ and $M_2$ macrophages. The protein product of SERPINE1, plasminogen activator inhibitor-1 (PAI-1) has been shown to inhibit MMP activity and therefore it is conceivable that down-regulation of SERPINE1 may promote the deposition of extracellular matrix [30].

Interestingly, the reduction in epithelial CLDN3, OCLN and TFF3 when cultured with $M_1$ and $M_2$ macrophages was not observed when co-cultured with $M_{2a}$, $M_{2b}$ or $M_{2c}$ macrophages, suggesting that these sub-types may rescue the de-differentiation. It was noteworthy that co-culture with $M_{2a}$, but not $M_{2b}$ or $M_{2c}$ macrophages, was associated with a marked decrease in Ki67 expression, together with reductions in fibronectin expression and soluble fibronectin in the cellular supernatants. Retinoic acid has been described to inhibit both fibronectin expression [31] and cellular proliferation [32] and has been shown to up-regulate MMP3 [33] and MMP14 [34] expression. Interestingly, of the three $M_2$ macrophage sub-types $M_{2b}$ and $M_{2c}$ macrophages released the most retinoic acid and expressed the highest levels of MMP-14 and MMP-3, the major fibronectin-degrading MMPs at physiological and acidic pH respectively [35]. Intracellular fibronectin was observed in epithelial cells on the leading edge of the wound after 24 hours when co-cultured with $M_{2b}$ and $M_{2c}$ macrophages, but was not observable in the intracellular, extracellular or soluble spaces after 72 hours. It could be speculated that rather than inhibit fibronectin expression, retinoic acid prevents integration of fibronectin into the extracellular matrix and that the intracellular fibronectin observed at 24 hours in the $M_{2b}$ and $M_{2c}$ co-cultures was subsequently degraded by MMP activity either at neutral pH or in the acidic environment found in the vicinity of activated macrophages [36].

In conclusion, we have generated an *in vitro* mechanical injury model in air-liquid interface differentiated primary human small airway epithelial cells that recapitulates many of the characteristics observed during epithelial wound injury in both human tissue and small animal models. Wound closure was initially associated with de-differentiation of the differentiated apical cells and rapid migration into the wound site, followed by proliferation of apical cells behind the wound edge, together with increases in FAK expression, fibronectin and reduction in PAI-1 which were associated with cell motility and extracellular matrix deposition. $M_1$ macrophages promoted FAK expression and both $M_1$ and $M_2$ macrophages promoted epithelial de-differentiation. Collectively these data highlight the complex nature of epithelial wound closure, the differential impact of macrophage sub-types on this process, and the heterogeneic and non-delineated function of these macrophages.

## Materials & methods

### Small airway epithelial cells

Clonetics™ Cells isolated from the 1 mm bronchiole area of normal human lung tissue (Lonza, donor #408031, 57 year old female; Caucasian; passage 2; seeding efficiency: 85%; doubling time: 37 h; viability: 79%) were cultured and differentiated according to the manufacturer's

instructions. As stated by Lonza, the cells were isolated from donated human tissue after obtaining permission for their use in research applications by informed consent or legal authorization. Briefly, after thawing, SAECs were seeded into a cell culture flask (day -8) in Clonetics S-ALI growth medium. On day -4, cells were trypsinised and seeded on transwell inserts (Corning, #3470) in 24 well plates at a density of $22x10^3$ cells/well. Airlift of cells was performed on day 0 by removing apical medium. Basolateral growth medium was substituted with differentiation medium (Clonetics S-ALI differentiation medium). Remaining growth factors on the apical side were removed by washing with prewarmed PBS. SAECs were differentiated for >4 weeks in an ALI until a pseudostratified epithelium, including mucus production and beating cilia, was observable under the microscope.

## Human monocyte derived macrophages

Peripheral blood mononuclear cells (PBMCs) were isolated from healthy human whole blood by means of density gradient centrifugation using Ficoll-Paque™ and a Leucosep Tube (Greiner Bio-One GmbH) according to manufacturer's instructions. Blood was donated by internal donors at the centre for occupational health at Boehringer Ingelheim in Biberach, the donors provided signed informed consent that allows use for scientific purposes. CD14 positive monocyte purification was performed by magnetic activated cell sorting (MACS) according to the manufacturer's instructions (Monocyte Isolation Kit II, Miltenyi Biotec) and seeded $1.5x10^5$ cells/$cm^2$ in XVIVO-10 medium (Lonza) into Thermo Scientific Nunc UpCell Surface cell culture plates. Medium was supplemented with either 100 ng/mL Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF) to induce an $M_1$ phenotype or Macrophage Colony Stimulating Factor (M-CSF) to induce an $M_2$ phenotype for 7 days. On day 7, differentiated $M_2$ macrophages were additionally stimulated with maturation factors for a further 3 days to induce sub-phenotype differentiation; either 20 ng/mL IL-4 and 20 ng/mL IL-13 ($M_{2a}$), 20 ng/mL LPS and 100 μg/mL immune complex ($M_{2b}$) or 20 ng/mL IL-10 ($M_{2c}$). Immune complexes were made by combining purified Beriglobin® (Intravenous immunoglobulin G (IVIG) preparation, CSL Behring) and Twinrix® (Hepatitis A/B vaccine preparation, GlaxoSmith Kline) with subsequent purification by means of size exclusion chromatography. Differentiated macrophages were detached from the UpCell Surface cell culture plates by letting the plates cool down to room temperature and cell concentration and viability was determined using the Countess™ Automated Cell Counter (Invitrogen).

## Scratch assay and live cell imaging

Injury simulation in differentiated SAECs was performed by manually scratching once across the cell layer on the transwell using a sterile 200 μL pipette tip (Eppendorf) taking care not to damage the membrane or to remove the collagen coating on the membrane. This would negatively influence the wound healing process. The size of the initial scratch area and subsequent cellular regrowth into the injured site was monitored using a CellIQ Analyser (Chip-Man Technologies Ltd.). Wound healing of scratched SAECs was monitored using Live Cell Imaging. Cells were cultivated at 37˚C with a gas flow of 14 mL per minute. Gas flow was repeatedly turned on for 15 minutes followed by 30 minutes without gas. Humidity was not regulated automatically by the system, but was achieved by filling residual wells and intermediate spaces of the 24 well plate with PBS. Cells were monitored for three days without medium exchange. To improve the visual contrast between cells and membrane pores, cells were labelled with 25 μM 5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester (CFSE; C1157, Thermo Fisher Scientific) for 20 minutes at 37˚C immediately after injury. Phase contrast images were

captured continuously and at every fourth cycle, the GFP channel was turned on to capture fluorescent images. Single images were stitched to grids and merged to videos.

## Macrophage-epithelial cell co-culture

To model the interaction between alveolar macrophages and airway epithelial cells, a co-culture system was developed. SAECs were cultivated as described above. Epithelial cells were scratched manually and the differentiated macrophages were added apically ($5x10^5$ cells per transwell). X-VIVO 10 medium (Lonza), which was supplemented with different stimulation factors, was added apically to promote macrophage differentiation into different phenotypes. Apical addition of the macrophage differentiation factors did not alter epithelial cell viability, growth or gene expression (data not shown).

## Transepithelial electrical resistance (TEER)

Transwells of ALI cultures were placed into a cellZscope (nanoAnalytics) for TEER measurement. Medium was added basolaterally (700 μL) and apically (200 μL) to enable impedance measurement. To maintain optimal culture conditions, the cellZscope was placed in a tissue culture incubator (37˚C / 5% $CO_2$). TEER was measured continually for up to 3 days.

## Immunofluorescent imaging

SAECs were fixed in 300 μL ice cold (1:1) Acetone-Methanol for 15 minutes. Cells were washed three times with 0.1% BSA in PBS and blocked with 5% BSA in PBS for at least one hour. The primary antibodies in 0.1% BSA in PBS were incubated on the cells for one hour, washed and secondary antibodies were incubated for one hour at 37˚C in the dark and washed once again. The transwell membrane was punched out with a biopsy punch, embedded with ProLong® Gold Antifade Mountant with DAPI (Thermo Fisher Scientific, P36935), placed on a glass slide, covered with a coverslip and examined under a confocal microscope (Zeiss Laser Scanning Microscope (LSM) 710). The following antibodies were used: Cytokeratin 5 Rabbit Monoclonal Antibody (EP1601Y; NB110-56916, Novus; 1:200); MUC5AC Mouse Monoclonal Antibody (45M1; MA5-12178, Thermo Fisher Scientific; 1:20); Ki-67 Mouse Monoclonal Antibody (180192Z, Thermo Fisher Scientific; 1:100); Fibronectin Mouse Monoclonal Antibody (3F12; MA5-14737, Thermo Fisher Scientific; 1:40); Vimentin Rabbit Polyclonal Antibody (S82; AP2739a, abgent; 1:400); FAK/PTK2 Rabbit Monoclonal Antibody (5H18L19; 701094, Thermo Fisher Scientific; 1:13); Smooth Muscle Actin Mouse Monoclonal Antibody (MA5-11547, Thermo Fisher Scientific; 1:400); Goat anti-Mouse IgG (H+L) Secondary Antibody, Alexa Fluor® 647 conjugate (1:400); and Donkey anti-Rabbit IgG (H+L) Secondary Antibody, Alexa Fluor® 568 conjugate (1:400). F-actin was stained with ActinGreen™ 488 ReadyProbes® Reagent (Thermo Fisher Scientific, R37110).

## Quantitative PCR

Cells were lysed with 200 μL buffer RLT (lysis buffer) for 10 minutes and frozen at -20˚C. RNA was isolated using the RNeasy Mini Kit by QIAGEN according to manufacturer's instructions. RNA concentration was determined using a NanoDrop 8000 spectrophotometer (Thermo Fisher Scientific). Isolated RNA (0.8μg) was reverse transcribed into cDNA using the High-Capacity cDNA Archive Kit (Applied Biosystems) in a RT-PCR cycler (Biometra TGradient Thermocycler) at 25˚C for 10 minutes, 37˚C for 120 minutes and 85˚C for 5 minutes. QuantiFast® Probe PCR + ROX Vial kit (QIAGEN) was used to prepare samples. Quantitative PCR was performed using dual-labeled probes (TaqMan® probes) with the Applied Biosystems

ViiA™ 7 real-time cycler using the following probe sets (all Thermo Fisher Scientific): 18S rRNA (4319413E), RNA polymerase II (Hs00172187_m1), Cytokeratin 5 (Hs00361185_m1), Mucin 5AC (Hs00873651_mH), Ki67 (Hs01032443_m1), Fibronectin 1 (Hs00365052_m1), Vimentin (Hs00185584_m1), Claudin 3 (Hs00265816_s1), Claudin 9 (Hs00253134_s1), Occludin (Hs00170162_m1), Forkhead Box J1 (FoxJ1; Hs00230964_m1), Secretoglobin Family 1A Member 1 (SCGB1A1; Hs00171092_m1), Trefoil Factor 3 (TFF3; Hs00902278_m1), Plasminogen Activator Inhibitor (SERPINE 1; Hs01126606_m1), Smooth Muscle Actin (Hs00426835_g1), Plasminogen Activator, Urokinase (Hs01547054_m1), and Urokinase Receptor (Hs00958880_m1).

### ELISA

Soluble human fibronectin was detected using a kit supplied by affymetrix eBiosciences (Human Fibronectin Platinum ELISA) and retinoic acid was measured using a kit supplied by cusabio (Human retinoic acid ELISA kit), both according to manufacturer's instructions. Absorbance was read on a spectrophotometer using 450 nm as the primary wavelength and 620 nm as reference wavelength (Spectramax M5e; Molecular Devices).

## Supporting information

**S1 Fig. TNFα production by different macrophage subtypes measured via MSD.**
(TIF)

**S2 Fig. TEER development of SAEC treated with 10 ng/mL TNF-α (orange line) or plain medium (black line) over 64h.**
(TIF)

**S1 Video. Wound healing response along the wound edge.**
(WMV)

**S2 Video. Influence of the collagen coating on the wound closure.**
(WMV)

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## Overall discussion of the thesis

The three publications presented herein describe *in vitro* and *in vivo* models that reflect aspects of IPF and COPD. We investigated the interactions of NTHi stimulated macrophages with the small airway epithelium, the influence of macrophages on re-epithelialization of small airway epithelial cells and the impact of cigarette smoke extract and H1N1 on bronchial epithelial cells form a COPD donor.

In publication #1 (Bucher et al., 2017), the individual or combined contribution of IL-1α and IL-1β and the role of IL-1R1 were investigated for the first time head-to-head in a recently described murine model where inflammation is triggered by CS and H1N1 mimicking aspects of an exacerbated inflammatory COPD phenotype (Bucher et al., 2016). We found that in particular combined treatment with IL-1α and IL-1β antibodies strongly reduced inflammation. We confirmed this in an *in vitro* model of air liquid interface differentiated diseased human bronchial epithelial cells infected with H1N1.

In publication #2 (Mang et al., 2018), for the first time we were able to show the importance of the IL-1 axis in bacterial/macrophage induction of epithelial permeability in a fully differentiated primary human macrophage-epithelial cell co-culture system. The COPD-relevant $M_{CSF2}$ (but not $M_{CSF1}$) MDMs stimulated with NTHi locally perturb the epithelial barrier to enable transepithelial chemokine/cytokine transit. This effect is mediated by NALP3, macrophage derived IL-1α, IL-1β, TNFα and by p38-MAPK.

In publication #3 (Gindele & Mang et al., 2017), we sought to model and characterise the pulmonary epithelial response to injury using mechanical scratching of primary human small airway epithelial cells in air-liquid interface culture as a test system. Furthermore, we aimed to explore the role of primary human macrophages differentiated *ex vivo* into different phenotypes on this process in a novel primary human cell co-culture system.

**SAEC/DHBE**

Small Airway Epithelial Cells and Bronchial Epithelial Cells cultivated in a fully differentiated, air-liquid interface culture were established and characterized during this thesis and used to obtain data in all three publications. They present a valuable tool to study pathological events that occur at and in the epithelium of the upper and lower lung.

The lower airway epithelium consists of basal cells, club cells, ciliated cells and mucus producing cells. All of these cells form tight junctions with each other to compose a tight barrier that protects the underlying tissue from being penetrated by pathogens or matter that is inspirated into the lung. The barrier integrity in the *in vitro* cultures can be measured as FITC-Dextrane permeability and transepithelial electrical resistance (TEER) which is a measurement of tight junction integrity.

IPF and COPD are both located in the lower airways where the inflammation and the structural changes are evident (Shaw et al., 2002). To confirm the findings in publication #1 in the mouse model of cigarette smoke and H1N1 which is associated with COPD exacerbations in humans (Seemungal et al., 2001), we used primary human bronchial epithelial cells from a COPD patient donor and infected these with H1N1. The blockade of IL-1β didn`t have an effect on the parameters tested whereas IL-1α blockade reversed TEER decrease as well as IL-6 and TNFα increase. The same effect could be observed for the combination of both antibodies.

The *in vivo* model showed a similar effect, however, the inhibition of both cytokines proved to be over-additive. This suggests a strong IL-1 component in these exacerbation models, that has been shown before (Pauwels et al., 2011, Piper et al., 2013, Finch et al., 2009, Churg et al., 2009).

It has been shown that the epithelium has the ability to reconstitute itself after injury, however, improper healing may occur which can lead to fibrosing of the tissue. This reconstitution not

only depends on the epithelial cells but is suspected to be influenced by macrophages as well (Lech and Anders, 2013). To test the impact of injuries on epithelial cells in publication #3, we used the established primary human small airway epithelial cells that were differentiated in an air-liquid interface. We introduced mechanical injuries to these cells, added different macrophage subtypes on top and followed different parameters like wound closure, expression of genes that are markers for migration, extracellular matrix and cell markers.

**Macrophages**

Different monocyte derived macrophage subtypes cultivated on top of Small Airway Epithelial Cells were established and characterized during this thesis. These hematopoietic cells were then used to obtain data in two of the three publications. They proved to be valuable as surrogates for alveolar macrophages that influence the inflammatory process (Mang et al., 2018) and the repair of mechanically introduced injury (Gindele & Mang et al., 2017).

Macrophages have been shown to patrol tissues, play a role in the defence against pathogens and influence the repair or remodelling of tissues (Murray and Wynn, 2011). For these studies we generated a previously published set of macrophage subtypes that resemble subtypes that have been associated with IPF and COPD (Lech and Anders, 2013, Barnes, 2004, Day et al., 2013). This set consists of $M_1$, activated $M_1$ ($M_{1.1}$), $M_2$, activated $M_2$ ($M_{2.1}$), $M_{2a}$, $M_{2b}$ and $M_{2c}$ and has been described as endpoints of differentiation phenotypes (Martinez and Gordon, 2014). The anti-inflammatory / pro-fibrotic types ($M_2$ subtypes) were used to determine their impact on the wound closure. The pro-inflammatory type ($M_1$) was used for the *in vitro* model of epithelial permeability to resemble the pro-inflammatory environment that is associated with a COPD exacerbation (Hurst et al., 2006). Macrophages activated by LPS/NTHi produced large amounts of cytokines in a subtype-dependent manner potentially contributing to the pro-inflammatory environment. In publication #2 the GM-CSF primed $M_1$ monocyte derived

macrophages showed higher pro-inflammatory cytokine (IL-1α, IL-1β, TNFα) production than M-CSF primed $M_2$. $M_1$ macrophages showed reduced expression of PPARγ after NTHi stimulation and a reduction of phagocytic activity and acidification of the medium. PPARγ is known to be lower in COPD patients and even further downregulated during COPD exacerbations (Zhao, 2011). GM-CSF is one of the cytokines upregulated in COPD which may bias macrophages towards a $M_1$ phenotype. This appears to be associated with a defective macrophage phenotype evidenced by reduced phagocytosis by macrophages derived from COPD patients associated with elevated GM-CSF levels (Chana et al., 2012, Day et al., 2014). The activated $M_1$ macrophage showed acidification of the medium and acidification of the extracellular space has been implicated in the activation of extracellular lysosomal cathepsins that can degrade extracellular matrix (Silver et al., 1988), a hallmark of emphysematous structural changes of the lung (Wolters and Chapman, 2000).

**Macrophages & SAEC**

Macrophages and epithelial cells are in close contact with each other in the lumen of the lung. We found a clear impact of macrophages on epithelial phenotypic characteristics during wound healing and also in the inflammatory response evoked by stimulating the co-cultures with NTHi. In publication #2 imaging of the macrophage - SAEC interaction revealed that the junctional protein ZO-1 dissociates from the cell junctions in the proximity of stimulated (but not unstimulated) macrophages, and not from junctions that are located distally from the stimulated macrophages. This might provide the means for cytokine transversal across the epithelium in order to signal to the extra-pulmonary compartments to recruit cells from, such as blood or bone marrow. A possibly analogous spatial connection between the site of tight junction disintegration, influx of neutrophils and peripheral macrophages has been demonstrated in dermal tissue where neutrophils preferentially extravasated in close proximity of perivascular macrophages (Abtin et al., 2014).

Along these lines, concomitant increase in cyto-/chemokine concentrations on the apical and basolateral side was observed in the experiments performed by us. In particular, the cyto-/chemokine concentrations on the basolateral side were dependent on the amount produced by macrophages on the apical side and the integrity of the tight junctions. A prime example is the macrophage derived CCL2. This observation was accompanied by Macrophage/NTHi dependent increase of FITC-Dextrane permeability. Increased cyto-/chemokine levels in serum (analogous to the basolateral compartment), broncho-alveolar lavage fluid, and sputum (both analogous to the apical compartment) have been associated with COPD and inhibition of these considered as treatment options (Caramori et al., 2014).

In publication #3 the apically added macrophages had distinct effects on the underlying injured epithelium. Here the wound closure was initially associated with de-differentiation of the epithelial cells and rapid migration into the site where cells had been removed by scratching. This was followed by proliferation of epithelial cells behind the wound edge, together with increases in FAK expression, fibronectin and reduction in PAI-1 which collectively facilitate cell motility and extracellular matrix deposition. $M_1$ macrophages promoted FAK expression and both $M_1$ and $M_2$ macrophages promoted epithelial de-differentiation. The data generated in this publication highlights the complex nature of epithelial wound closure, the differential impact of macrophage sub-types on this process, and the heterogeneic and non-delineated function of macrophages in general.

**Il-1 axis**

Prominent cytokines that are associated with COPD are IL-1α and IL-1β (Pauwels et al., 2011) and there are several lines of evidence (including our ALI studies with epithelial cells from COPD donors) that indicate that the IL-1 pathway is active in patients with COPD.

Furthermore, it has been demonstrated that the pro-inflammatory IL-1 family members IL-1α and IL-1β are increased in patients with stable COPD and during exacerbations (Bafadhel et al., 2011, Botelho et al., 2011, Gessner et al., 2005, Pauwels et al., 2011, Sapey et al., 2009). Previous *in vitro* studies with human primary cells suggest a plethora of roles of IL-1 family members in the inflammatory process (Garlanda et al., 2013). Although these cytokines have been shown to be secreted by various cell types, the results presented herein suggest that the main source of IL-1 is macrophages rather than epithelial cells, at least at low concentrations of bacterial stimuli.

In publication #1 we found that individual neutralization of IL-1α in CS/H1N1 challenged mice had significant suppressive effects. However, individual neutralization of IL-1β did not provide significant effects. Remarkably, the combined neutralization of IL-1α and IL-1β had additive or over-additive effects. This data significantly extends a previous report investing the role of IL-1α in a related smoke/viral challenge model (Botelho et al., 2011). Furthermore, we found that treatment of H1N1-infected primary human bronchial epithelial cells obtained from a donor with COPD with IL-1α antibodies resulted in significant protection from H1N1-induced loss in TEER and in significantly reduced levels of IL-6 and TNFα. An antibody against IL-1β, however, had no protective effect. This confirms our *in vivo* findings.

In publication #2, NTHi stimulated co-cultures of SAEC and GM-CSF stimulated macrophages, together with antibodies that neutralize IL-1α and IL-1β or the IL-1 receptor antagonist IL-1RA showed that the IL-1 pathway is important in the perturbation of epithelial barrier integrity. IL-1α inhibition alone did not result in significant attenuation of TEER decrease caused by NTHi stimulated macrophages. Whereas, blocking IL-1β yielded around 62 % inhibition. Strikingly. the combination of both antibodies showed full inhibition of the TEER decrease. A similar effect of IL-1 on TEER has been studied in various tissues where tight junctions play a role such as the blood-brain-barrier and blood-testis-barrier (de Vries et al., 1996, Lie et al., 2011).

A highly interesting and analogous example is the work of Herold et al. where they demonstrated that resident alveolar macrophages disrupted the epithelial barrier through IL-1β. This lead to influx of exudate macrophages into the lung in a murine model of gram-negative pneumonitis (Herold et al., 2011). However, contrary to our data, the effect was attributed to apoptosis within the epithelium - which we didn`t observe - rather than tight junction disruption. Furthermore, the attenuation was attributed to IL-1RA secreted by immigrating exudate macrophages. IL-1RA should block the IL-1β effect but would also mask the possible effects of other IL-1R activators like IL-1α making it difficult to attribute the effect to a single one of the activators of IL-1R. In our model of NTHi stimulated macrophages, the clinically approved IL-1 antagonist IL-1RA (Anakinra) resulted in a 42 % inhibition of TEER decrease. This rather low level of effect is unexpected but resembles the finding of Finch et al. who achieved 60 % macrophage influx inhibition in their short term LPS mouse model (Finch et al., 2009). Our work, the work done by Finch et al. and Harold et al. shows that there is a strong spatial dependence of where the pro inflammatory challenge occurs and where the resulting cell influx is derived from. This particularly reinforces our assumption that it is the macrophage derived IL-1 and the IL-1 pathway in the epithelial cells that is important for epithelial barrier disruption. These findings and many others validate pharmacological intervention in the IL-1 pathway as a therapeutic concept in COPD, which has been attempted with approaches like antibodies, e.g. Canakinumab.

Canakinumab is an IL-1β blocking antibody that, despite the clear involvement of IL-1 in COPD did not reach its clinical efficacy endpoints in COPD. (Rogliani et al., 2015)

Our results shown in publication #1, the H1N1 model (Bucher et al., 2017) and in publication #2, the co-culture model (Mang et al., 2018) may explain why this particular antibody did not have the desired effect. By assuming that IL-1α and IL-1β both need to be inhibited to achieve

meaningful effects, the potential reduction achieved by solely blocking IL-1β may be a possible explanation of the lack of clinical efficacy in this study.

The NALP3 inflammasome is another interesting target as it is crucial for the generation of IL-1β. It is activated mainly by products of damaged cells like extracellular ATP and activates the IL-1β processing Caspase-1. The activity of this pathway has been associated with COPD (Faner et al., 2016, De Nardo et al., 2014). Inhibition of the NALP3 inflammasome showed reduced IL-1β but not IL-1α on the apical side of the co-cultures and reversed TEER drop which underpins the observation that blocking IL-1β is beneficial for the integrity of the epithelial barrier.

TLR4 inhibition showed reduction of apical cytokines by almost 100 % and inhibition of TEER drop by 62 % suggesting that TLR4 is the main receptor for the NTHi-mediated effects in this co-culture. There appear to be no clinical trials that have tested TLR4 inhibitors to treat COPD exacerbations. Nevertheless, TLR4 inhibition has been suggested as a treatment option (Zuo et al., 2015).

Inhibition of p38-MAPK was associated with no change of apical CCL2 compared to the stimulated control but reduction of CCL2 on the basolateral side and TEER decrease was reversed. This suggests that p38-MAPK plays a role in the integrity of epithelial tight junctions. However, IL-1β did not show this differential distribution suggesting a partial role of p38-MAPK in macrophages as well. Clinical evaluation of p38-MAPK inhibitors has been shown to be an interesting approach in an LPS challenge model where it reduced neutrophil counts in sputum (Singh et al., 2015) and improved lung function in COPD patients (MacNee et al., 2013). However, most clinically developed p38-MAPK inhibitors have been discontinued or their status of development is unknown, possibly due to undesirable side effects (Norman, 2015). We measured CCL2 as a surrogate since it is mainly produced by apical macrophages in our

model and therefore can only be found at very low levels on the basolateral side until the tight junctions are compromised. Therefore, we measured the basolateral CCL2 as an indicator of epithelial permeability for macrophage produced chemokines. Furthermore, it has been shown to be relevant in recruiting cells into the lung lumen (Herold et al., 2011).

**Microinjury**

A contributing factor of the pathogenesis of IPF may be the introduction of lesions and micro-injuries to the lower airway epithelium (Selman et al., 2001) which, if inappropriately repaired may lead to fibrotic foci (Chambers, 2008). It has been assumed that macrophages play an important role in the resolution and/or pathogenesis of IPF (Lech and Anders, 2013). Therefore, we introduced scratches to human small airway epithelial cell cultures and added different subtypes of macrophages to determine the effects they have on the epithelial wound healing.

Monitoring of epithelial wound repair revealed a highly migratory process. Cells in the vicinity of the scratch were observed to migrate into the cell-free space, a motion that appeared to start from several "hot spot" origins, which is consistent with the observations of Erjefält *et al.* (Erjefalt et al., 1995). This was consistent with the recovery of TEER, which was not complete until approximately 48 hours after injury. This presumably is a measure of the recovery of epithelial integrity and the generation of new tight junctions, despite the fact that the expression of both OCLN and CLDN3 were still down-regulated 72 hours after injury (although mRNA levels as a labile precursor do not necessarily reflect protein levels). Interestingly, the basal cells did not stain positively for the proliferation marker Ki67. However, the proliferating cells appeared to reside in the apical layer behind the leading edge of the wound, but only at later time points. This was confirmed by Ki67 mRNA levels that were elevated 48 and even more so at 72 hours post-injury. Furthermore, basal cells were initially observed on the leading edge of the wound at early time points, but not thereafter. This would indicate that the initial physical

wound closure and recovery of epithelial barrier integrity is predominantly facilitated by migration of differentiated cells. These cells migrate from the apical layer into the damaged area, and once barrier integrity is restored, distal apical cells proliferate to replenish cell numbers. This observation is contrary to reports that suggest that basal cells play a more dominant role in barrier recovery following oxidative (Pardo-Saganta et al., 2015, Rock et al., 2011) or chemical (Hong et al., 2004, Ghosh et al., 2013) damage. It could be speculated that this apparent discrepancy is in response to different types of injury. And indeed, small animal models of mechanical epithelial injury appear to indicate that an initial key event is the dedifferentiation and flattening of cells that border the lesion. These cells then appear to migrate inwards and over the denuded area to restore the barrier function of the epithelium (Wilhelm, 1953, Keenan et al., 1982c, Keenan et al., 1982b, Keenan et al., 1982a, Kim et al., 1997, Shimizu et al., 1994, McDowell et al., 1987).

Interestingly, an intact collagen-coated membrane appears to be vital for the initial migratory process, although cells appear to be able to eventually cross a damaged membrane as cells on the opposing wound edge come closer. This suggests that perhaps a soluble mediator may be involved or responsible.

FAK is a kinase that is closely associated with the cytoskeleton, is associated with cell motility and epithelial-mesenchymal transition (EMT) (Schaller, 2010), an event linked with COPD (Wang et al., 2013) and IPF (Chapman, 2011).

In the immunocytochemistry, FAK was up-regulated on the leading edge of the wound 24 hours after injury which is consistent with the motility observed in the time-lapse videos. We also observed de-differentiation in the apical cells following injury, as described by the down-regulation of FOXJ1 (ciliated cell marker), SCGB1A1 (club cell marker), TFF3 (columnar epithelial cell marker) and ACTA2 (smooth muscle actin). This is consistent with the

observations reported in the small animal models. We also observed a concomitant increase in both mRNA and protein levels of fibronectin following injury. Initially this was apparently intracellular on the leading edge of the wound, but later also apparently deposited extracellularly. Generally, accumulation of extracellular matrix proteins such as collagen or fibronectin is a physiological response to injury (Agren and Werthen, 2007) and its effect can be seen in the inhibition of migration if matrix proteins are missing after damaging the membrane. Both soluble and insoluble fibronectin have been shown to increase alveolar epithelial wound healing in an *in vitro* model, by increasing cell motility and spreading, but apparently not by cellular proliferation (Garat et al., 1996).

To determine the effect of macrophages on the damaged epithelium, we added the different subtypes to the culture. $M_1$ macrophages promoted FAK expression suggesting enhanced migration and both $M_1$ and $M_2$ macrophages promoted epithelial de-differentiation. $M_1$ macrophages were associated with a greater down-regulation of epithelial Muc5AC, which has also been demonstrated to be down-regulated during EMT (Johnson et al., 2013), but down-regulation was even more pronounced in the co-culture with $M_2$ macrophages. SERPINE1 expression was up-regulated following injury, but down-regulated in the presence of both $M_1$ and $M_2$ macrophages. The protein product of SERPINE1, plasminogen activator inhibitor-1 (PAI-1) has been shown to inhibit MMP activity and therefore it is conceivable that down-regulation of SERPINE1 may promote the deposition of extracellular matrix (Flevaris and Vaughan, 2017).

Interestingly, the reduction in epithelial CLDN3, OCLN and TFF3 when cultured with $M_1$ and $M_2$ macrophages was not observed when co-cultured with $M_{2a}$, $M_{2b}$ or $M_{2c}$ macrophages, suggesting that these sub-types may rescue the de-differentiation and possibly have an impact on barrier formation. It was noteworthy that co-culture with $M_{2a}$, but not $M_{2b}$ or $M_{2c}$

macrophages, was associated with a marked decrease in Ki67 expression, together with reductions in fibronectin expression and soluble fibronectin.

Retinoic acid has been described to inhibit both fibronectin expression (Scita et al., 1996) and cellular proliferation (Cui et al., 2016) and has been shown to up-regulate MMP3 (Yasuhara et al., 2010) and MMP14 (Jurzak et al., 2008) expression. Interestingly, of the three $M_2$ macrophage sub-types $M_{2b}$ and $M_{2c}$ macrophages released the most retinoic acid and expressed the highest levels of MMP-14 and MMP-3, the major fibronectin-degrading MMPs at physiological and acidic pH respectively (Zhang et al., 2012). Intracellular fibronectin was observed in epithelial cells on the leading edge of the wound after 24 hours when co-cultured with $M_{2b}$ and $M_{2c}$ macrophages, but was not observable in the intracellular, extracellular or soluble spaces after 72 hours. It could be speculated that retinoic acid has an effect on fibronectin integration into the extracellular matrix rather than its expression and that the intracellular fibronectin observed at 24 hours in the $M_{2b}$ and $M_{2c}$ co-cultures was subsequently degraded by MMP activity either at neutral pH or in the acidic environment found in the vicinity of activated macrophages (Silver et al., 1988).

Taken together, we were able to demonstrate the importance of the IL-1 pathway in an *in vivo* and *in vitro* setting of viral or bacterial induced exacerbations of COPD. For the first time we show that $M_1$ but not $M_2$ MDMs added apically to differentiated small airway epithelium, stimulated with heat-inactivated NTHi locally perturb the epithelial barrier. This enables transepithelial chemo- and cytokine transit. The effect is mediated by NALP3, macrophage derived IL-1α, IL-1β, TNFα and by p38-MAPK. Furthermore, our work supports and extends previous findings that IL-1α might be a key player driving CS/virus induced airway inflammation and that IL-1β is a major player in the bacterial induced airway inflammation. Nevertheless, combination of both anti- IL-1α and anti-IL-1β treatment showed over-additive effects in both models. Furthermore, we demonstrated that macrophages may not only have a

major role in exacerbating COPD but also have an influence on the healing process of the lung epithelium depending on their subtype. These models not only presented interesting insights in the biology of these diseases but also a valuable tool to study pharmacological intervention that might lead to the development of new drugs for patients.

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**Declaration according to §6 paragraph 2 Nr. 7 of the promotion order**

I hereby declare that this thesis entitled CHARACTERISATION OF PATHWAYS INVOLVED IN BACTERIAL OR VIRAL INDUCED EXACERBATIONS OF AIRWAY DISEASES has been composed solely by myself and that it has not been submitted, in whole or in part, in any previous application for a degree.

Except where stated otherwise by reference or acknowledgement, the work presented is entirely my own.

I have not received support by mediation or consulting services (promotion consultants or other). No one has received direct or indirect payment for work that is related to the thesis. I have conducted the research for this thesis at the following institution: Boehringer Ingelheim GmbH & Co. KG; Biberach an der Riss, Germany.

I declare that I have given this information to the best of my knowledge.

Place, Date_____ Signature_____

## Publications:

### Publications that are part of the thesis:

Publication #1:

Bucher, H., **Mang, S.**, Keck, M., Przibilla, M., Lamb, D. J., Schiele, F., Wittenbrink, M., Fuchs, K., Jung, B., Erb, K. J. & Peter, D. 2017. *Neutralization of both IL-1alpha/IL-1beta plays a major role in suppressing combined cigarette smoke/virus-induced pulmonary inflammation in mice*. Pulm Pharmacol Ther, 44, 96-105.

Publication #2:

**Mang S.**, Braun A., Pairet N., Lamb D.J., 2018. *Importance of the IL-1 Axis in Haemophilus influenzae-stimulated $M_1$ macrophages Driving Transepithelial Signaling*. Am J Respir Cell Mol Biol, 58, 412-415

Publication #3

Gindele, J. A.*, **Mang, S.***, Pairet, N., Christ, I., Gantner, F., Schymeinsky, J. & Lamb, D. J. 2017. *Opposing effects of in vitro differentiated macrophages sub-type on epithelial wound healing*. PLoS One, 12, e0184386.

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### Further publications that are not part of the thesis:

Fischer, S., Buck, T., Wagner, A., Ehrhart, C., Giancaterino, J., **Mang, S.**, Schad, M., Mathias, S., Aschrafi, A., Handrick, R. & Otte, K. 2014. *A functional high-content miRNA screen identifies miR-30 family to boost recombinant protein production in CHO cells.* Biotechnology Journal, 9: 1279–1292

**Mang, S.**, Bucher, H. & Nickolaus, P. 2016. *Development of a Scintillation Proximity Assay (SPA) Based, High Throughput Screening Feasible Method for the Identification of PDE12 Activity Modulators*. Curr Drug Discov Technol., 13: 144-151

Pairet, N., **Mang, S.**, Fois, G., Keck, M., Kühnbach, M., Gindele, J.A., Dietl, P., Frick, M., Lamb, D.J., 2018. *TRPV4 inhibition attenuates stretch–induced inflammatory cellular responses and lung barrier dysfunction during mechanical ventilation.* PLoS One, 13, e0196055.

Pairet, N., **Mang, S.**, Kiechle, T., Laufhäger, N., Dietl, P., Lamb, D.J., 2018. *Differential modulation of cellular function by TRPV4 agonists is mediated by apoptosis and/or necrosis*. Biochemistry and Biophysics Reports, under review

## Conference contributions:

C. Hesse, **S. Mang**, P. Braubach, D. Jonigk, G. Warnecke, O. Pfennig, H.-G. Fieguth, A. Braun and K. Sewald, 2016, *Ex vivo induction of features displaying early onset pulmonary fibrosis in PCLS.* DZL Annual Meeting (Hannover)

C. Hesse, **S. Mang**, H.-G. Hoymann, M. Niehof, P. Braubach, D. Jonigk, G. Warnecke, O. Pfennig, H.-G. Fieguth, A. Braun, and K. Sewald, 2016, *Identification of pro-fibrotic biomarkers in precision-cut lung slices (PCLS).* EUSAAT (Linz)

C. Hesse, **S. Mang**, H.-G. Hoymann, M. Niehof, P. Braubach, D. Jonigk, G. Warnecke, O. Pfennig, H.-G. Fieguth, A. Braun, and K. Sewald, 2016, *Induction of pro-fibrotic biomarkers in precision-cut lung slices (PCLS).* ERS (London):

C. Hesse, **S. Mang**, H.-G. Hoymann, M. Niehof, P. Braubach, D. Jonigk, M. Kühnel, G. Warnecke, O. Pfennig, H.-G. Fieguth, A. Braun, and K. Sewald, 2016, *Identification of pro-fibrotic biomarkers in precision-cut lung slices (PCLS).* DGP Herbsttreffen (Hannover)

**S. Mang**, A. Braun, D.J. Lamb, 2017, *COPD patient isolated nontypeable Haemophilus influenzae stimulates $M_1$ macrophages to produce Interleukins 1α and 1β which synergistically disrupt epithelial barrier function and drive trans-epithelial signaling*. Fraunhofer seminar Translational Airway Research "Models of Lung Disease" (Hannover)

C. Hesse, **S. Mang**, P. Braubach, D. Jonigk, G. Warnecke, O. Pfennig, H.-G. Fieguth, A. Braun and K. Sewald, 2017, *Ex vivo induction of features displaying early onset pulmonary fibrosis in PCLS*. Fraunhofer seminar Translational Airway Research "Models of Lung Disease" (Hannover)